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(54) Title: METHODS AND COMPOSITIONS FOR STIMULATING BONE CELLS			
(57) Abstract Disclosed are methods, compositions, kits and devices for use in transferring nucleic acids into bone cells <i>in situ</i> and/or for stimulating bone progenitor cells. Type II collagen and, particularly, osteotropic genes, are shown to stimulate bone progenitor cells and to promote bone growth, repair and regeneration <i>in vivo</i> . Gene transfer protocols are disclosed for use in transferring various nucleic acid materials into bone, as may be used in treating various bone-related diseases and defects including fractures, osteoporosis, osteogenesis, imperfecta and in connection with bone implants.			

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DESCRIPTIONMethods and Compositions for Stimulating Bone Cells

5 The present application is a continuation-in-part of
U.S. Serial Number 08/316,650, filed September 30, 1994;
which is a continuation-in-part of U.S. Serial Number
08/199,780, filed February 18, 1994; the entire text and
10 figures of which disclosures are specifically
incorporated herein by reference without disclaimer. The
United States government has certain rights in the
present invention pursuant to Grant HL-41926 from the
National Institutes of Health.

15 1. Field of the Invention

 The present invention relates generally to the field
of bone cells and tissues. More particularly, certain
embodiments concern the transfer of genetic material into
20 bone and other embodiments concern type II collagen. In
certain examples, the invention concerns the use of type
II collagen and nucleic acids to stimulate bone growth,
repair and regeneration. Methods, compositions, kits and
devices are provided for transferring an osteotropic gene
25 into bone progenitor cells, which is shown to stimulate
progenitor cells and to promote increased bone formation
in vivo.

2. Description of the Related Art

30 Defects in the process of bone repair and
regeneration are linked to the development of several
human diseases and disorders, e.g., osteoporosis and
osteogenesis imperfecta. Failure of the bone repair
35 mechanism is, of course, also associated with significant
complications in clinical orthopaedic practice, for
example, fibrous non-union following bone fracture,

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implant interface failures and large allograft failures. The lives of many individuals would be improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

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Naturally, any new technique to stimulate bone repair would be a valuable tool in treating bone fractures. A significant portion of fractured bones are still treated by casting, allowing natural mechanisms to effect wound repair. Although there have been advances in fracture treatment in recent years, including improved devices, the development of new processes to stimulate, or complement, the wound repair mechanisms would represent significant progress in this area.

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A very significant patient population that would benefit from new therapies designed to promote fracture repair, or even prevent or lessen fractures, are those patients suffering from osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age.

An estimated 20-25 million people are at increased risk for fracture because of site-specific bone loss. The cost of treating osteoporosis in the United States is currently estimated to be in the order of \$10 billion per year. Demographic trends, i.e., the gradually increasing age of the US population, suggest that these costs may increase 2-3 fold by the year 2020 if a safe and

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effective treatment is not found.

The major focus of current therapies for osteoporosis is fracture prevention, not fracture repair. This is an important consideration, as it is known that significant morbidity and mortality are associated with prolonged bed rest in the elderly, especially those who have suffered hip fracture. New methods are clearly needed for stimulating fracture repair, thus restoring mobility in these patients before the complications arise.

Osteogenesis imperfecta (OI) refers to a group of inherited connective tissue diseases characterized by bone and soft connective tissue fragility (Byers and Steiner, 1992; Prockop, 1990). Males and females are affected equally, and the overall incidence is currently estimated to be 1 in 5,000-14,000 live births. Hearing loss, dentinogenesis imperfecta, respiratory insufficiency, severe scoliosis and emphysema are just some of the conditions that are associated with one or more types of OI. While accurate estimates of the health care costs are not available, the morbidity and mortality associated with OI certainly result from the extreme propensity to fracture (OI types I-IV) and the deformation of abnormal bone following fracture repair (OI types II-IV) (Bonadio and Goldstein, 1993). The most relevant issue with OI treatment is to develop new methods by which to improve fracture repair and thus to improve the quality of life of these patients.

The techniques of bone reconstruction, such as is used to reconstruct defects occurring as a result of trauma, cancer surgery or errors in development, would also be improved by new methods to promote bone repair. Reconstructive methods currently employed, such as using autologous bone grafts, or bone grafts with attached soft

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tissue and blood vessels, are associated with significant drawbacks of both cost and difficulty. For example, harvesting a useful amount of autologous bone is not easily achieved, and even autologous grafts often become
5 infected or suffer from resorption.

The process of bone repair and regeneration resembles the process of wound healing in other tissues. A typical sequence of events includes; hemorrhage; clot
10 formation; dissolution of the clot with concurrent removal of damaged tissues; ingrowth of granulation tissue; formation of cartilage; capillary ingrowth and cartilage turnover; rapid bone formation (callus tissue); and, finally, remodeling of the callus into cortical and
15 trabecular bone. Therefore, bone repair is a complex process that involves many cell types and regulatory molecules. The diverse cell populations involved in fracture repair include stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts,
20 and osteoclasts.

Regulatory factors involved in bone repair are known to include systemic hormones, cytokines, growth factors, and other molecules that regulate growth and
25 differentiation. Various osteoinductive agents have been purified and shown to be polypeptide growth-factor-like molecules. These stimulatory factors are referred to as bone morphogenetic or morphogenic proteins (BMPs), and have also been termed osteogenic bone inductive proteins
30 or osteogenic proteins (OPs). Several BMP (or OP) genes have now been cloned, and the common designations are BMP-1 through BMP-8. New BMPs are in the process of discovery. Although the BMP terminology is widely used, it may prove to be the case that there is an OP

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counterpart term for every individual BMP (Alper, 1994).

BMPs 2-8 are generally thought to be osteogenic, although BMP-1 is a more generalized morphogen (Shimell et al., 1991). BMP-3 is also called osteogenin (Luyten et al., 1989) and BMP-7 is also called OP-1 (Ozkaynak et al., 1990). BMPs are related to, or part of, the transforming growth factor- β (TGF- β) superfamily, and both TGF- β 1 and TGF- β 2 also regulate osteoblast function (Seitz et al., 1992). Several BMP (or OP) nucleotide sequences and polypeptides have been described in U.S. Patents, e.g., 4,795,804; 4,877,864; 4,968,590; 5,108,753; including, specifically, BMP-1 disclosed in U.S. Patent 5,108,922; BMP-2A (currently referred to as BMP-2) in U.S. Patents 5,166,058 and 5,013,649; BMP-2B (currently referred to as BMP-4) disclosed in U.S. Patent 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; BMP-7 in 5,108,753 and 5,141,905; and OP-1, COP-5 and COP-7 in 5,011,691.

Other growth factors or hormones that have been reported to have the capacity to stimulate new bone formation include acidic fibroblast growth factor (Jingushi et al., 1990); estrogen (Boden et al., 1989); macrophage colony stimulating factor (Horowitz et al., 1989); and calcium regulatory agents such as parathyroid hormone (PTH) (Raisz and Kream, 1983).

Several groups have investigated the possibility of using bone stimulating proteins and polypeptides, particularly recombinant BMPs, to influence bone repair in vivo. For example, recombinant BMP-2 has been employed to repair surgically created defects in the mandible of adult dogs (Toriumi et al., 1991), and high doses of this molecule have been shown to functionally repair segmental defects in rat femurs (Yasko et al., 1992). Chen and colleagues showed that a single

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application of 25-100 mg of recombinant TGF- β 1 adjacent to cartilage induced endochondral bone formation in the rabbit ear full thickness skin wounds (Chen et al., 1991). It has also been reported that an application of

5 TGF- β 1 in a 3% methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck et al., 1991).

10 However, there are many drawbacks associated with these type of treatment protocols, not least the expensive and time-consuming purification of the recombinant proteins from their host cells. Also, polypeptides, once administered to an animal are more

15 unstable than is generally desired for a therapeutic agent, and they are susceptible to proteolytic attack. Furthermore, the administration of recombinant proteins can initiate various inhibitive or otherwise harmful immune responses. It is clear, therefore, that a new

20 method capable of promoting bone repair and regeneration in vivo would represent a significant scientific and medical advance with immediate benefits to a large number of patients. A method readily adaptable for use with a variety of matrices and bone-stimulatory genes would be

25 particularly advantageous.

SUMMARY OF THE INVENTION

30 The present invention overcomes one or more of these and other drawbacks inherent in the prior art by providing novel methods, compositions and devices for use in transferring nucleic acids into bone cells and tissues, and for promoting bone repair and regeneration.

35 Certain embodiments of the invention rest, generally, with the inventors' surprising finding that nucleic acids can be effectively transferred to bone progenitor cells

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in vivo and that, in certain embodiments, the transfer of an osteotropic gene stimulates bone repair in an animal.

The invention, in general terms, thus concerns
5 methods, compositions and devices for transferring a nucleic acid segment into bone progenitor cells or tissues. The methods of the invention generally comprise contacting bone progenitor cells with a composition comprising a nucleic acid segment in a manner effective
10 to transfer the nucleic acid segment into the cells. The cells may be cultured cells or recombinant cells maintained in vitro, when all that is required is to add the nucleic acid composition to the cells, e.g., by adding it to the culture media.

15 Alternatively, the progenitor cells may be located within a bone progenitor tissue site of an animal, when the nucleic acid composition would be applied to the site in order to effect, or promote, nucleic acid transfer
20 into bone progenitor cells in vivo. In transferring nucleic acids into bone cells within an animal, a preferred method involves first adding the genetic material to a bone-compatible matrix and then using the resultant matrix to contact an appropriate tissue site
25 within the animal. The "resultant" matrix may, in certain embodiments, be referred to as a matrix impregnated with genetic material, or it may take the form of a matrix-nucleic acid mixture, or even conjugate.

30 An extremely wide variety of genetic material can be transferred to bone progenitor cells or tissues using the compositions and methods of the invention. For example, the nucleic acid segment may be DNA (double or single-stranded) or RNA (e.g., mRNA, tRNA, rRNA); it may also be
35 a "coding segment", i.e., one that encodes a protein or polypeptide, or it may be an antisense nucleic acid molecule, such as antisense RNA that may function to

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disrupt gene expression. The nucleic acid segments may thus be genomic sequences, including exons or introns alone or exons and introns, or coding cDNA regions, or in fact any construct that one desires to transfer to a bone progenitor cell or tissue. Suitable nucleic acid segments may also be in virtually any form, such as naked DNA or RNA, including linear nucleic acid molecules and plasmids; functional inserts within the genomes of various recombinant viruses, including viruses with DNA genomes and retroviruses; and any form of nucleic acid segment, plasmid or virus associated with a liposome or a gold particle, the latter of which may be employed in connection with the gene gun technology.

The invention may be employed to promote expression of a desired gene in bone cells or tissues and to impart a particular desired phenotype to the cells. This expression could be increased expression of a gene that is normally expressed (*i.e.*, "over-expression"), or it could be used to express a gene that is not normally associated with bone progenitor cells in their natural environment. Alternatively, the invention may be used to suppress the expression of a gene that is naturally expressed in such cells and tissues, and again, to change or alter the phenotype. Gene suppression may be a way of expressing a gene that encodes a protein that exerts a down-regulatory function, or it may utilize antisense technology.

1. Bone Progenitor Cells and Tissues

In certain embodiments, this invention provides advantageous methods for using genes to stimulate bone progenitor cells. As used herein, the term "bone progenitor cells" refers to any or all of those cells that have the capacity to ultimately form, or contribute to the formation of, new bone tissue. This includes

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various cells in different stages of differentiation, such as, for example, stem cells, macrophages, fibroblasts, vascular cells, osteoblasts, chondroblasts, osteoclasts, and the like. Bone progenitor cells also
5 include cells that have been isolated and manipulated in vitro, e.g., subjected to stimulation with agents such as cytokines or growth factors or even genetically engineered cells. The particular type or types of bone progenitor cells that are stimulated using the methods
10 and compositions of the invention are not important, so long as the cells are stimulated in such a way that they are activated and, in the context of *in vivo* embodiments, ultimately give rise to new bone tissue.

15 The term "bone progenitor cell" is also used to particularly refer to those cells that are located within, are in contact with, or migrate towards (i.e., "home to"), bone progenitor tissue and which cells directly or indirectly stimulate the formation of mature
20 bone. As such, the progenitor cells may be cells that ultimately differentiate into mature bone cells themselves, i.e., cells that "directly" form new bone tissue. Cells that, upon stimulation, attract further progenitor cells or promote nearby cells to differentiate
25 into bone-forming cells (e.g., into osteoblasts, osteocytes and/or osteoclasts) are also considered to be progenitor cells in the context of this disclosure - as their stimulation "indirectly" leads to bone repair or regeneration. Cells affecting bone formation indirectly
30 may do so by the elaboration of various growth factors or cytokines, or by their physical interaction with other cell types. Although of scientific interest, the direct or indirect mechanisms by which progenitor cells

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stimulate bone or wound repair is not a consideration in practicing this invention.

Bone progenitor cells and bone progenitor tissues
5 may be cells and tissues that, in their natural
environment, arrive at an area of active bone growth,
repair or regeneration (also referred to as a wound
repair site). In terms of bone progenitor cells, these
10 may also be cells that are attracted or recruited to such
an area. These may be cells that are present within an
artificially-created osteotomy site in an animal model,
such as those disclosed herein. Bone progenitor cells
may also be isolated from animal or human tissues and
maintained in an *in vitro* environment. Suitable areas of
15 the body from which to obtain bone progenitor cells are
areas such as the bone tissue and fluid surrounding a
fracture or other skeletal defect (whether or not this is
an artificially created site), or indeed, from the bone
marrow. Isolated cells may be stimulated using the
20 methods and compositions disclosed herein and, if
desired, be returned to an appropriate site in an animal
where bone repair is to be stimulated. In such cases,
the nucleic-acid containing cells would themselves be a
form of therapeutic agent. Such *ex vivo* protocols are
25 well known to those of skill in the art.

In important embodiments of the invention, the bone
progenitor cells and tissues will be those cells and
tissues that arrive at the area of bone fracture or
30 damage that one desires to treat. Accordingly, in
treatment embodiments, there is no difficulty associated
with the identification of suitable target progenitor
cells to which the present therapeutic compositions
should be applied. All that is required in such cases is
35 to obtain an appropriate stimulatory composition, as
disclosed herein, and contact the site of the bone
fracture or defect with the composition. The nature of

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this biological environment is such that the appropriate cells will become activated in the absence of any further targeting or cellular identification by the practitioner.

5 Certain methods of the invention involve, generally, contacting bone progenitor cells with a composition comprising one or more osteotropic genes (with or without additional genes, proteins or other biomolecules) so as to promote expression of said gene in said cells. As
10 outlined above, the cells may be contacted *in vitro* or *in vivo*. This is achieved, in the most direct manner, by simply obtaining a functional osteotropic gene construct and applying the construct to the cells. The present
15 inventors surprisingly found that there are no particular molecular biological modifications that need to be performed in order to promote effective expression of the gene in progenitor cells. Contacting the cells with DNA, e.g., a linear DNA molecule, or DNA in the form of a
20 plasmid or other recombinant vector, that contains the gene of interest under the control of a promoter, along with the appropriate termination signals, is sufficient to result in uptake and expression of the DNA, with no further steps necessary.

25 In preferred embodiments, the process of contacting the progenitor cells with the osteotropic gene composition is conducted *in vivo*. Again, a direct consequence of this process is that the cells take up and express the gene and that they, without additional steps,
30 function to stimulate bone tissue growth, repair or regeneration.

 An assay of an osteoinductive gene may be conducted using the bone induction bioassay of Sampath and Reddi
35 (1981; incorporated herein by reference). This is a rat bone formation assay that is routinely used to evaluate the osteogenic activity of bone inductive factors.

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However, for analyzing the effects of osteotropic genes on bone growth, one is generally directed to use the novel osteotomy model disclosed herein.

5 2. Osteotropic Genes

As used herein, the terms "osteotropic and osteogenic gene" are used to refer to a gene or DNA coding region that encodes a protein, polypeptide or peptide that is capable of promoting, or assisting in the promotion of, bone formation, or one that increases the rate of primary bone growth or healing (or even a gene that increases the rate of skeletal connective tissue growth or healing). The terms promoting, inducing and stimulating are used interchangeably throughout this text to refer to direct or indirect processes that ultimately result in the formation of new bone tissue or in an increased rate of bone repair. Thus, an osteotropic gene is a gene that, when expressed, causes the phenotype of a cell to change so that the cell either differentiates, stimulates other cells to differentiate, attracts bone-forming cells, or otherwise functions in a manner that ultimately gives rise to new bone tissue.

25 In using the new osteotomy model of the invention, an osteotropic gene is characterized as a gene that is capable of stimulating proper bone growth in the osteotomy gap to any degree higher than that observed in control studies, e.g., parallel studies employing an irrelevant marker gene such as β -galactosidase. This stimulation of "proper bone growth" includes both the type of tissue growth and the rate of bone formation. In using the model with a 5 mm osteotomy gap, an osteotropic gene is generally characterized as a gene that is capable of promoting or inducing new bone formation, rather than abnormal bone fracture repair, i.e., fibrous non-union. 35 In using the 2 mm osteotomy gap, one may characterize

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osteotropic genes as genes that increase the rate of primary bone healing as compared to controls, and more preferably, genes capable of stimulating repair of the osteotomy defect in a time period of less than nine weeks.

In general terms, an osteotropic gene may also be characterized as a gene capable of stimulating the growth or regeneration of skeletal connective tissues such as, e.g., tendon, cartilage, and ligament. Thus, in certain embodiments, the methods and compositions of the invention may be employed to stimulate the growth or repair of both bone tissue itself and also of skeletal connective tissues.

A variety of osteotropic genes are now known, all of which are suitable for use in connection with the present invention. Osteotropic genes and the proteins that they encode include, for example, systemic hormones, such as parathyroid hormone (PTH) and estrogen; many different growth factors and cytokines; chemotactic or adhesive peptides or polypeptides; molecules such as activin (U.S. Patent 5,208,219, incorporated herein by reference); specific bone morphogenetic proteins (BMPs); and even growth factor receptor genes.

Examples of suitable osteotropic growth factors include those of the transforming growth factor (TGF) gene family, including TGFs 1-3, and particularly TGF- β 1, TGF- β 2 and TGF- β 3, (U.S. Patents 4,886,747 and 4,742,003, incorporated herein by reference), with TGF- α (U.S. Patent 5,168,051, incorporated herein by reference) also being of possible use; and also fibroblast growth factors (FGF), previously referred to as acidic and basic FGF and now referred to as FGF1-9; granulocyte/macrophage colony stimulating factor (GM-CSF); epidermal growth factor (EGF); platelet derived growth factor (PDGF); insulin-

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like growth factors (IGF), including IGF-I and IGF-II; and leukemia inhibitory factor (LIF), also known as HILDA and DIA. Any of the above or other related genes, or DNA segments encoding the active portions of such proteins, may be used in the novel methods and compositions of the invention.

Certain preferred osteotropic genes and DNA segments are those of the TGF superfamily, such as TGF- β 1, TGF- β 2, TGF- β 3 and members of the BMP family of genes. For example, several BMP genes have been cloned that are ideal candidates for use in the nucleic acid transfer or delivery protocols of the invention. Suitable BMP genes are those designated BMP-2 through BMP-12. BMP-1 is not considered to be particularly useful at this stage.

There is considerable variation in the terminology currently employed in the literature in referring to these genes and polypeptides. It will be understood by those of skill in the art that all BMP genes that encode an active osteogenic protein are considered for use in this invention, regardless of the differing terminology that may be employed. For example, BMP-3 is also called osteogenin and BMP-7 is also called OP-1 (osteogenic protein-1). It is likely that the family of factors termed OP(s) is as large as that termed BMP(s), and that these terms, in fact, describe the same set of molecules (Alper, 1994).

The DNA sequences for several BMP (or OP) genes have been described both in scientific articles and in U.S. Patents such as 4,877,864; 4,968,590; 5,108,753. Specifically, BMP-1 sequences are disclosed in U.S. Patent 5,108,922; BMP-2A (currently referred to as BMP-2) in U.S. Patents 5,166,058 and 5,013,649; BMP-2B (currently referred to as BMP-4) disclosed in U.S. Patent 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6

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in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all incorporated herein by reference). The article by Wozney et al., (1988; incorporated herein by reference) is considered to be particularly useful for describing BMP molecular clones and their activities. DNA sequences encoding the osteogenic proteins designated OP-1, COP-5 and COP-7 are also disclosed in U.S. Patent 5,011,691.

All of the above issued U.S. Patents are incorporated herein by reference and are intended to be used in order to supplement the present teachings regarding the preparation of BMP and OP genes and DNA segments that express osteotropic polypeptides. As disclosed in the above patents, and known to those of skill in the art, the original source of a recombinant gene or DNA segment to be used in a therapeutic regimen need not be of the same species as the animal to be treated. In this regard, it is contemplated that any recombinant PTH, TGF or BMP gene may be employed to promote bone repair or regeneration in a human subject or an animal, e.g., a horse. Particularly preferred genes are those from human, murine and bovine sources, in that such genes and DNA segments are readily available, with the human or murine forms of the gene being most preferred for use in human treatment regimens. Recombinant proteins and polypeptides encoded by isolated DNA segments and genes are often referred to with the prefix "r" for recombinant and "rh" for recombinant human. As such, DNA segments encoding rBMPs, such as rhBMP-2 or rhBMP-4, are contemplated to be particularly useful in connection with this invention.

The definition of a "BMP gene", as used herein, is a gene that hybridizes, under relatively stringent hybridization conditions (see, e.g., Maniatis et al., 1982), to DNA sequences presently known to include BMP gene sequences.

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To prepare an osteotropic gene segment or cDNA one may follow the teachings disclosed herein as also the teachings of any of patents or scientific documents specifically referenced herein. Various nucleotide sequences encoding active BMPs are disclosed in U.S. Patents 5,166,058, 5,013,649, 5,116,738, 5,106,748, 5,187,076, 5,108,753 and 5,011,691, each incorporated herein by reference. By way of example only, U.S. Patent 5,166,058, teaches that hBMP-2 is encoded by a nucleotide sequence from nucleotide #356 to nucleotide #1543 of the sequence shown in Table II of the patent. One may thus obtain a hBMP-2 DNA segment using molecular biological techniques, such as polymerase chain reaction (PCR™) or screening a cDNA or genomic library, using primers or probes with sequences based on the above nucleotide sequence. The practice of such techniques is a routine matter for those of skill in the art, as taught in various scientific articles, such as Sambrook et al., (1989), incorporated herein by reference. Certain documents further particularly describe suitable mammalian expression vectors, e.g., U.S. Patent 5,168,050, incorporated herein by reference.

Osteotropic genes and DNA segments that are particularly preferred for use in certain aspects of the present compositions and methods are the TGF, PTH and BMP genes. TGF genes are described in U.S. Patents 5,168,051; 4,886,747 and 4,742,003, each incorporated herein by reference. TGF α may not be as widely applicable as TGF β , but is proposed for use particularly in applications involving skeletal soft tissues. The PTH gene, or a DNA segment encoding the active fragment thereof, such as a DNA segment encoding a polypeptide that includes the amino acids 1-34 (hPTH1-34; Hendy et al., 1981; incorporated herein by reference) is another

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preferred gene; as are the BMP genes termed BMP-4 and BMP-2, such as the gene or cDNA encoding the murine BMP-4 disclosed herein.

5 It is also contemplated that one may clone further genes or cDNAs that encode an osteotropic protein or polypeptide. The techniques for cloning DNA molecules, i.e., obtaining a specific coding sequence from a DNA library that is distinct from other portions of DNA, are
10 well known in the art. This can be achieved by, for example, screening an appropriate DNA library, as disclosed in Example XV herein, which relates to the cloning of a wound healing gene. The screening procedure may be based on the hybridization of oligonucleotide
15 probes, designed from a consideration of portions of the amino acid sequence of known DNA sequences encoding related osteogenic proteins. The operation of such screening protocols are well known to those of skill in the art and are described in detail in the scientific
20 literature, for example, in Sambrook et al., (1989), incorporated herein by reference.

Osteotropic genes with sequences that vary from those described in the literature are also encompassed by
25 the invention, so long as the altered or modified gene still encodes a protein that functions to stimulate bone progenitor cells in any direct or indirect manner. These sequences include those caused by point mutations, those due to the degeneracies of the genetic code or naturally
30 occurring allelic variants, and further modifications that have been introduced by genetic engineering, i.e., by the hand of man.

Techniques for introducing changes in nucleotide
35 sequences that are designed to alter the functional properties of the encoded proteins or polypeptides are well known in the art, e.g., U.S. Patent 4,518,584,

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incorporated herein by reference, which techniques are also described in further detail herein. Such modifications include the deletion, insertion or substitution of bases, and thus, changes in the amino acid sequence. Changes may be made to increase the osteogenic activity of a protein, to increase its biological stability or half-life, to change its glycosylation pattern, and the like. All such modifications to the nucleotide sequences are encompassed by this invention.

It will, of course, be understood that one or more than one osteotropic gene may be used in the methods and compositions of the invention. The nucleic acid delivery methods may thus entail the administration of one, two, three, or more, osteotropic genes. The maximum number of genes that may be applied is limited only by practical considerations, such as the effort involved in simultaneously preparing a large number of gene constructs or even the possibility of eliciting a significant adverse cytotoxic effect. The particular combination of genes may be two or more distinct BMP genes; or it may be such that a growth factor gene is combined with a hormone gene, e.g., a BMP gene and a PTH gene; a hormone or growth factor gene may even be combined with a gene encoding a cell surface receptor capable of interacting with the polypeptide product of the first gene.

In using multiple genes, they may be combined on a single genetic construct under control of one or more promoters, or they may be prepared as separate constructs of the same or different types. Thus, an almost endless combination of different genes and genetic constructs may be employed. Certain gene combinations may be designed to, or their use may otherwise result in, achieving synergistic effects on cell stimulation and bone growth,

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any and all such combinations are intended to fall within the scope of the present invention. Indeed, many synergistic effects have been described in the scientific literature, so that one of ordinary skill in the art
5 would readily be able to identify likely synergistic gene combinations, or even gene-protein combinations.

It will also be understood that, if desired, the nucleic segment or gene could be administered in
10 combination with further agents, such as, e.g., proteins or polypeptides or various pharmaceutically active agents. So long as genetic material forms part of the composition, there is virtually no limit to other components which may also be included, given that the
15 additional agents do not cause a significant adverse effect upon contact with the target cells or tissues. The nucleic acids may thus be delivered along with various other agents, for example, in certain embodiments one may wish to administer an angiogenic factor, and/or
20 an inhibitor of bone resorption, as disclosed in U.S. Patents 5,270,300 and 5,118,667, respectively, each incorporated herein by reference.

3. Gene Constructs and DNA Segments

25 As used herein, the terms "gene" and "DNA segment" are both used to refer to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a gene or DNA segment encoding an
30 osteotropic gene refers to a DNA segment that contains sequences encoding an osteotropic protein, but is isolated away from, or purified free from, total genomic DNA of the species from which the DNA is obtained. Included within the term "DNA segment", are DNA segments
35 and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phage, retroviruses, adenoviruses, and the like.

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The term "gene" is used for simplicity to refer to a functional protein or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences and cDNA sequences.

5 "Isolated substantially away from other coding sequences" means that the gene of interest, in this case, an osteotropic gene, forms the significant part of the coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-
10 occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions, such as sequences encoding leader peptides or targeting
15 sequences, later added to the segment by the hand of man.

This invention provides novel ways in which to utilize various known osteotropic DNA segments and recombinant vectors. As described above, many such
20 vectors are readily available, one particular detailed example of a suitable vector for expression in mammalian cells is that described in U.S. Patent 5,168,050, incorporated herein by reference. However, there is no requirement that a highly purified vector be used, so
25 long as the coding segment employed encodes a osteotropic protein and does not include any coding or regulatory sequences that would have a significant adverse effect on bone progenitor cells. Therefore, it will also be understood that useful nucleic acid sequences may include
30 additional residues, such as additional non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

35 After identifying an appropriate osteotropic gene or DNA molecule, it may be inserted into any one of the many vectors currently known in the art, so that it will

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direct the expression and production of the osteotropic protein when incorporated into a bone progenitor cell. In a recombinant expression vector, the coding portion of the DNA segment is positioned under the control of a promoter. The promoter may be in the form of the promoter which is naturally associated with an osteotropic gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an osteotropic gene in its natural environment. Such promoters may include those normally associated with other osteotropic genes, and/or promoters isolated from any other bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in bone progenitor cells.

The use of recombinant promoters to achieve protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook et al., (1989). The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level or regulated expression of the introduced DNA segment. The currently preferred promoters are those such as CMV, RSV LTR, the SV40 promoter alone, and the SV40 promoter in combination with various enhancer elements.

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Osteotropic genes and DNA segments may also be in the form of a DNA insert which is located within the genome of a recombinant virus, such as, for example a recombinant adenovirus, adeno-associated virus (AAV) or retrovirus. In such embodiments, to place the gene in contact with a bone progenitor cell, one would prepare the recombinant viral particles, the genome of which includes the osteotropic gene insert, and simply contact the progenitor cells or tissues with the virus, whereby the virus infects the cells and transfers the genetic material.

In certain preferred embodiments, one would impregnate a matrix or implant material with virus by soaking the material in recombinant virus stock solution, e.g., for 1-2 hours, and then contact the bone progenitor cells or tissues with the resultant, impregnated matrix. Cells then penetrate, or grow into, the matrix, thereby contacting the virus and allowing viral infection which leads to the cells taking up the desired gene or cDNA and expressing the encoded protein.

In other preferred embodiments, one would form a matrix-nucleic acid admixture, whether using naked DNA, a plasmid or a viral vector, and contact the bone progenitor cells or tissues with the resultant admixed matrix. The matrix may then deliver the nucleic acid into the cells following disassociation at the cell surface, or in the immediate cellular environment. Equally, the matrix admixture itself, especially a particle- or fiber-DNA admixture, may be taken up by cells to provide subsequent intracellular release of the genetic material. The matrix may then be extruded from the cell, catabolized by the cell, or even stored within the cell. The molecular mechanism by which a bone-compatible matrix achieves transfer of DNA to a cell is immaterial to the practice of the present invention.

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4. Bone-Compatible Matrices

In certain preferred embodiments, the methods of the invention involved preparing a composition in which the
5 osteotropic gene, genes, DNA segments, or cells already incorporating such genes or segments, are associated with, impregnated within, or even conjugated to, a bone-compatible matrix, to form a "matrix-gene composition" and the matrix-gene composition is then placed in contact
10 with the bone progenitor cells or tissue. The matrix may become impregnated with a gene DNA segment simply by soaking the matrix in a solution containing the DNA, such as a plasmid solution, for a brief period of time of
15 anywhere from about 5 minutes or so, up to and including about two weeks.

Matrix-gene compositions are all those in which genetic material is adsorbed, absorbed, impregnated, conjugated to, or otherwise generally maintained in
20 contact with the matrix. "Maintained in contact with the matrix" means that an effective amount of the nucleic acid composition should remain functionally associated with the matrix until its transfer to the bone progenitor cell or its release in the bone tissue site.

25 The type of matrix that may be used in the compositions, devices and methods of the invention is virtually limitless, so long as it is a "bone-compatible matrix". This means that the matrix has all the features
30 commonly associated with being "biocompatible", in that it is in a form that does not produce a significant adverse, allergic or other untoward reaction when administered to an animal, and that it is also suitable for placing in contact with bone tissue. A "significant"
35 adverse effect is one that exceeds the normally accepted side-effects associated with any given therapy.

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"Bone-compatible", as used herein, means that the matrix (and gene) does not produce a significant adverse or untoward reaction when placed in contact with bone. In certain embodiments, when electing to use a particular
5 bone compatible matrix, one may, optionally, take various other factors into consideration, for example, the capacity of the matrix to provide a structure for the developing bone, its capacity to be resorbed into the body after the bone has been repaired, and such like.
10 However, these properties are not required to practice the invention and are merely exemplary of the factors that may be considered.

In other embodiments, one may also consider the
15 likelihood that the matrix will be transported into the cell, e.g., by active or passive membrane transport. Where such transport and subsequent nucleic acid release is contemplated, other properties of the matrix and gene may be assessed in optimizing the matrix-gene
20 formulation. For example, adenovirus vectors may provide for advantageous DNA release in such embodiments. Matrices that are readily metabolized in the cytoplasm would also likely be preferred in such embodiments. Matrices that are later released from the cell, and
25 preferably, also removed from the surrounding tissue area, would be another preferred form of matrix for use in such embodiments.

The choice of matrix material will differ according
30 to the particular circumstances and the site of the bone that is to be treated. Matrices such as those described in U.S. Patent 5,270,300 (incorporated herein by reference) may be employed. Physical and chemical characteristics, such as, e.g., biocompatibility,
35 biodegradability, strength, rigidity, interface properties, and even cosmetic appearance, may be considered in choosing a matrix, as is well known to

- 25 -

those of skill in the art. Appropriate matrices will deliver the gene composition and, in certain circumstances, may be incorporated into a cell, or may provide a surface for new bone growth, i.e., they may act
5 as an *in situ* scaffolding through which progenitor cells may migrate.

A particularly important aspect of the present invention is its use in connection with orthopaedic
10 implants and interfaces and artificial joints, including implants themselves and functional parts of an implant, such as, e.g., surgical screws, pins, and the like. In preferred embodiments, it is contemplated that the metal surface or surfaces of an implant or a portion thereof,
15 such as a titanium surface, will be coated with a material that has an affinity for nucleic acids, most preferably, with hydroxyl apatite, and then the coated-metal will be further coated with the gene or nucleic acid that one wishes to transfer. The available chemical
20 groups of the absorptive material, such as hydroxyl apatite, may be readily manipulated to control its affinity for nucleic acids, as is known to those of skill in the art.

25 In certain embodiments, non-biodegradable matrices may be employed, such as sintered hydroxylapatite, aluminates, other bioceramic materials and metal materials, particularly titanium. A suitable ceramic delivery system is that described in U.S. Patent
30 4,596,574, incorporated herein by reference. Polymeric matrices may also be employed, including acrylic ester polymers, lactic acid polymers, and polylactic polyglycolic acid (PLGA) block copolymers, have been disclosed (U.S. Patent 4,526,909, U.S. Patent 4,563,489,
35 Simons et al., 1992, and Langer and Folkman, 1976, respectively, each incorporated herein by reference).

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In certain embodiments, it is contemplated that a biodegradable matrix will likely be most useful. A biodegradable matrix is generally defined as one that is capable of being resorbed into the body. Potential
5 biodegradable matrices for use in connection with the compositions, devices and methods of this invention include, for example, biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxylapatite, PLGA block copolymers, polyanhydrides,
10 matrices of purified proteins, and semi-purified extracellular matrix compositions.

One preferred group of matrices are collagenous matrices, including those obtained from tendon or dermal
15 collagen, e.g., type I collagen, which is generally prepared from dermis; those obtained from cartilage, such as type II collagen; and various other types of collagen. Collagens may be obtained from a variety of commercial sources, e.g., Sigma that supplies type II collagen
20 obtained from bovine trachea; and Collagen Corporation. Collagen matrices may also be prepared as described in U.S. Patents 4,394,370 and 4,975,527, each incorporated herein by reference.

25 The various collagenous materials may also be in the form of mineralized collagen. One preferred mineralized collagenous material is that termed UltraFiber™, obtainable from Norian Corp. (Mountain View, CA). U.S. Patent 5,231,169, incorporated herein by reference,
30 describes the preparation of mineralized collagen through the formation of calcium phosphate mineral under mild agitation in situ in the presence of dispersed collagen fibrils. Such a formulation may be employed in the

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context of delivering a nucleic acid segment to a bone tissue site.

Certain other preferred collagenous materials are those based upon type II collagen. Type II collagen preparations have been discovered to have the surprising and advantageous property of, absent any osteotropic gene, being capable of stimulating bone progenitor cells. Prior to the present invention, it was thought that type II collagen only had a structural role in the cartilage extracellular matrix and the present finding that type II collagen is actually an osteoconductive/osteoinductive material is unexpected. The present invention thus contemplates the use of a variety of type II collagen preparations as gene transfer matrices or bone cell stimulants, either with or without DNA segments, including native type II collagen, as prepared from cartilage, and recombinant type II collagen.

PLGA block copolymers may also be employed as gene transfer matrices. Such polymers have been shown to readily incorporate DNA, are commercially available, non-toxic, and hydrolyze at defined rates, (i.e. they facilitate the sustained release of pharmaceutical agents). PLGA block copolymers have two particular advantageous properties in that, first, they exhibit reversible thermal gelation, and second, may be combined with other agents that allow for radiographic visualization.

5. Nucleic Acid Transfer Embodiments

Once a suitable matrix-gene composition has been prepared or obtained, all that is required to deliver the osteotropic gene to bone progenitor cells within an animal is to place the matrix-gene composition in contact

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with the site in the body in which one wishes to promote bone growth. This may be achieved by physically positioning the matrix-gene composition in contact with the body site, or by injecting a syringeable form of the
5 matrix-gene composition into the appropriate area.

The matrix-gene composition may be applied to a simple bone fracture site that one wishes to repair, an area of weak bone, such as in a patient with
10 osteoporosis, or a bone cavity site that one wishes to fill with new bone tissue. Bone cavities may arise as a result of an inherited disorder, birth defect, or may result following dental or periodontal surgery or after the removal of an osteosarcoma.

15 The use of PLGA and like compounds as matrices allows the matrix-DNA composition to be syringeable, which is achieved by, generally, admixing the matrix-gene composition with a pluronic agent. The resultant matrix-gene-pluronic may be stored within a thermal-jacket
20 syringe, maintained at a temperature of about 4°C, immediately prior to administration to the body. In this temperature and environment, the composition will be a liquid. Following insertion into the body, the
25 composition will equilibrate towards body temperature, and in so-doing will form a gelatinous matrix.

The above phenomenon is termed "reversible thermal gelation", and this allows for a controlled rate of
30 gelation to be achieved. The manner of using pluronic agents in this context will be known to those of skill in the art in light of the present disclosure. Matrix-gene-pluronic compositions may also be admixed, or generally associated with, an imaging agent so that the present
35 gene transfer technology may be used in imaging modalities. In these cases, the attending physician or veterinarian will be able to monitor the delivery and

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positioning of the matrix-gene composition. Many safe and effective imaging agents, such as the radiographic compound calcium phosphate, are available that may be used in conjunction with fluoroscopy, or even with
5 tomography, to image the body or tissue site while the composition is being delivered.

Where an image of the tissue site is to be provided, one will desire to use a detectable imaging agent, such
10 as a radiographic agent, or even a paramagnetic or radioactive agent. Many radiographic diagnostic agents are known in the art to be useful for imaging purposes, including e.g., calcium phosphate.

15 In the case of paramagnetic ions, examples include chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium
20 (III) and erbium (III), with gadolinium being generally preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to, lanthanum (III), gold (III), lead (II), and especially bismuth (III).

25 Although not generally preferred, radioactive isotopes are not excluded and may be used for imaging purposes if desired. Suitable ions include iodine¹³¹, iodine¹²³, technetium^{99m}, indium¹¹¹, rhenium¹⁸⁸, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰, iodine¹²⁵ and astatine²¹¹.

30 The amount of gene construct that is applied to the matrix and the amount of matrix-gene material that is applied to the bone tissue will be determined by the attending physician or veterinarian considering various
35 biological and medical factors. For example, one would wish to consider the particular osteotropic gene and matrix, the amount of bone weight desired to be formed,

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the site of bone damage, the condition of the damaged bone, the patient's or animal's age, sex, and diet, the severity of any infection, the time of administration and any further clinical factors that may affect bone growth, such as serum levels of various factors and hormones. The suitable dosage regimen will therefore be readily determinable by one of skill in the art in light of the present disclosure, bearing in mind the individual circumstances.

In treating humans and animals, progress may be monitored by periodic assessment of bone growth and/or repair, e.g., using X-rays. The therapeutic methods and compositions of the invention are contemplated for use in both medical and veterinary applications, due to the lack of species specificity in bone inductive factors. In particular, it is contemplated that domestic, farm and zoological animals, as well as thoroughbred horses, would be treatable using the nucleic acid transfer protocols disclosed herein.

The present methods and compositions may also have prophylactic uses in closed and open fracture reduction and also in the improved fixation of artificial joints. The invention is applicable to stimulating bone repair in congenital, trauma-induced, or oncologic resection-induced craniofacial defects, and also is useful in the treatment of periodontal disease and other tooth repair processes and even in cosmetic plastic surgery. The matrix-gene compositions and devices of this invention may also be used in wound healing and related tissue repair, including, but not limited to healing of burns, incisions and ulcers.

The present invention also encompasses DNA-based compositions for use in cellular transfer to treat bone defects and disorders. The compositions of the invention

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generally comprise an osteotropic gene in association with a bone-compatible matrix, such as type II collagen, wherein the composition is capable of stimulating bone growth, repair or regeneration upon administration to, or
5 implantation within, a bone progenitor tissue site of an animal. The osteotropic gene or genes may be any of those described above, with TGF- α (for soft skeletal tissues), TGF- β 1, TGF- β 2, TGF- β 3, PTH, BMP-2 and BMP-4 genes being generally preferred. Likewise, irrespective
10 of the choice of gene, the bone-compatible matrix may be any of those described above, with biodegradable matrices such as collagen and, more particularly, type II collagen, being preferred.

15 In still further embodiments, the present invention concerns osteotropic devices, which devices may be generally considered as molded or designed matrix-gene compositions. The devices of the invention naturally
20 comprise a bone-compatible matrix in which an osteotropic gene is associated with the matrix. The combination of genes and matrix components is such that the device is capable of stimulating bone growth or healing when implanted in an animal. The devices may be of virtually
25 any size or shape, so that their dimensions are adapted to fit a bone fracture or bone cavity site in the animal that is to be treated, allowing the fracture join and/or bone regrowth to be more uniform. Other particularly
30 contemplated devices are those that are designed to act as an artificial joint. Titanium devices and hydroxylapatite-coated titanium devices will be preferred in certain embodiments. Parts of devices in combination with an osteotropic nucleic acid segment, such as a DNA-coated screw for an artificial joint, and the like, also
35 fall within the scope of the invention.

Therapeutic kits comprising, in suitable container means, a bone compatible matrix, such as type II collagen

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or a PLGA block polymer, and an osteotropic gene form another aspect of the invention. Such kits will generally contain a pharmaceutically acceptable formulation of the matrix and a pharmaceutically acceptable formulation of an osteotropic gene, such as PTH, BMP, TGF- β , FGF, GMCSF, EGF, PDGF, IGF or a LIF gene. Currently preferred genes include PTH, TGF- β 1, TGF- β 2, TGF- β 3, and BMP-4 genes.

10 The kits may comprise a single container means that contains both the biocompatible matrix and the osteotropic gene. The container means may, if desired, contain a pharmaceutically acceptable sterile syringeable matrix, having associated with it, the osteotropic gene composition and, optionally, a detectable label or
15 imaging agent. The syringeable matrix-DNA formulation may be in the form of a gelatinous composition, e.g., a type II collagen-DNA composition, or may even be in a more fluid form that nonetheless forms a gel-like
20 composition upon administration to the body. In these cases, the container means may itself be a syringe, pipette, or other such like apparatus, from which the matrix-DNA material may be applied to a bone tissue site or wound area. However, the single container means may
25 contain a dry, or lyophilized, mixture of a matrix and osteotropic gene composition, which may or may not require pre-wetting before use.

 Alternatively, the kits of the invention may
30 comprise distinct container means for each component. In such cases, one container would contain the osteotropic gene, either as a sterile DNA solution or in a lyophilized form, and the other container would include the matrix, which may or may not itself be pre-wetted
35 with a sterile solution, or be in a gelatinous, liquid or other syringeable form.

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The kits may also comprise a second or third container means for containing a sterile, pharmaceutically acceptable buffer, diluent or solvent. Such a solution may be required to formulate either the DNA component, the matrix component, both components separately, or a pre-mixed combination of the components, into a more suitable form for application to the body, e.g., a more gelatinous form. It should be noted, however, that all components of a kit could be supplied in a dry form (lyophilized), which would allow for "wetting" upon contact with body fluids. Thus, the presence of any type of pharmaceutically acceptable buffer or solvent is not a requirement for the kits of the invention. The kits may also comprise a second or third container means for containing a pharmaceutically acceptable detectable imaging agent or composition.

The container means will generally be a container such as a vial, test tube, flask, bottle, syringe or other container means, into which the components of the kit may placed. The matrix and gene components may also be aliquoted into smaller containers, should this be desired. The kits of the present invention may also include a means for containing the individual containers in close confinement for commercial sale, such as, e.g., injection or blow-molded plastic containers into which the desired vials or syringes are retained.

Irrespective of the number of containers, the kits of the invention may also comprise, or be packaged with, an instrument for assisting with the placement of the ultimate matrix-gene composition within the body of an animal. Such an instrument may be a syringe, pipette, forceps, or any such medically approved delivery vehicle.

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6. Type II Collagen as an Osteoconductive/inductive Material

5 The present invention also provides methods for stimulating bone progenitor cells, as may be applied, in certain circumstances, to promote new bone formation, or to stimulate wound-healing. As such, the bone progenitor cells that are the targets of the invention may also be termed "wound healing bone progenitor cells". Although
10 the function of wound healing itself may not always be required to practice all aspects of the invention, and although a mechanistic understanding is not necessary to practice the invention, it is generally thought that the wound healing process does operate during execution of
15 the invention.

To stimulate a bone progenitor cell in accordance with these aspects of the invention, generally one would contact a bone progenitor cell with a composition
20 comprising a biologically effective amount of type II collagen. Although preparations of crushed bone and mineralized collagen have been shown to be osteoconductive, this property has not previously been ascribed to type II collagen. The present inventors have
25 found that type II collagen alone is surprisingly effective at promoting new bone formation, it being able to bridge a 5 mm osteotomy gap in only eight weeks in all animals tested (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, and FIG.
30 8C).

The forms of type II collagen that may be employed in this invention are virtually limitless. For example, type II collagen may be purified from hyaline cartilage
35 of bovine trachea, or as isolated from diarthrodial joints or growth plates. Purified type II collagen is commercially available and may be purchased from, e.g., Sigma Chemical Company, St. Louis, MO. Any form of

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recombinant type II collagen may also be employed, as may be obtained from a type II collagen-expressing recombinant host cell, including bacterial, yeast, mammalian, and insect cells. One particular example of a recombina
5 nt type II collagen expression system is a yeast cell that includes an expression vector that encodes type II collagen, as disclosed herein in Example VI.

The type II collagen used in the invention may, if
10 desired, be supplemented with additional minerals, such as calcium, e.g., in the form of calcium phosphate. Both native and recombinant type II collagen may be supplemented by admixing, adsorbing, or otherwise associating with, additional minerals in this manner.
15 Such type II collagen preparations are clearly distinguishable from the types of "mineralized collagen" previously described, e.g., in U.S. Patent 5,231,169 that describes the preparation of mineralized total collagen fibrils.

20 An object of this aspect of the invention is to provide a source of osteoconductive matrix material, that may be reproducibly prepared in a straightforward and cost-effective manner, and that may be employed, with or
25 without an osteotropic gene segment, to stimulate bone progenitor cells. Recombinant type II collagen was surprisingly found to satisfy these criteria. Although clearly not required for effective results, the combination of native or recombinant type II collagen
30 with mineral supplements, such as calcium, is encompassed by this invention.

A biologically effective amount of type II collagen is an amount of type II collagen that functions to
35 stimulate a bone progenitor cell, as described herein. By way of example, one measure of a biologically effective amount is an amount effective to stimulate bone

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progenitor cells to the extent that new bone formation is evident. In this regard, the inventors have shown that 10 mg of lyophilized collagen functions effectively to close a 5 mm osteotomy gap in three weeks. This
5 information may be used by those of skill in the art to optimize the amount of type II collagen needed for any given situation.

Depending on the individual case, the artisan would,
10 in light of this disclosure, readily be able to calculate an appropriate amount, or dose, of type II collagen for stimulating bone cells and promoting bone growth. In terms of small animals or human subjects, suitable effective amounts of collagen include between about 1 mg
15 and about 500 mg, and preferably, between about 1 mg and about 100 mg, of lyophilized type II collagen per bone tissue site. Of course, it is likely that there will be variations due to, e.g., individual responses, particular tissue conditions, and the speed with which bone
20 formation is required. While 10 mg were demonstrated to be useful in the illustrative example, the inventors contemplate that 1, 5, 10, 15, 20, 30, 40, 50, 75, 100, 125, 150, 200, 300 mg, and the like, may be usefully employed for human patients and small animals. Of
25 course, any values within these contemplated ranges may be useful in any particular case.

Naturally, one of the main variables to be accounted for is the amount of new bone that needs to be generated
30 in a particular area or bone cavity. This can be largely a function of the size of the animal to be treated, e.g., a cat or a horse. Therefore, there is currently no upper limit on the amount of type II collagen, or indeed on the amount of any matrix-gene composition, that can be
35 employed in the methods of the invention, given careful supervision by the practitioner.

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In contacting or applying type II collagen, with or without a DNA segment, to bone progenitor cells located within a bone progenitor tissue site of an animal, bone tissue growth will be stimulated. Thus, bone cavity sites and bone fractures may be filled and repaired.

The use of type II collagen in combination with a nucleic acid segment that encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells is preferred, as described above. Nucleic acid segments that comprise an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or a chemotactic factor gene are preferred, with PTH, TGF- β and BMP genes being most preferred. The genes function subsequent to their transfer into, and expression in, bone progenitor cells of the treated animal, thus promoting bone growth.

Although type II collagen alone is effective, its combined use with an osteotropic gene segment may prove to give synergistic and particularly advantageous effects. Type II collagen, whether native or recombinant, may thus also be formulated into a therapeutic kit with an osteotropic gene segment, in accordance with those kits described herein above. This includes the use of single or multiple container means, and combination with any medically approved delivery vehicle, including, but not limited to, syringes, pipettes, forceps, additional diluents, and the like.

30

BRIEF DESCRIPTION OF THE DRAWINGS

The drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings

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in combination with the detailed description of specific embodiments presented herein.

FIG. 1. A model of DNA therapy for bone repair.

5

FIG. 2A. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the method of creating osteotomy and placing gene-activated matrix *in situ*.

10

FIG. 2B. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the method of fracturing repair cells where blood vessels grow into the gene-activated matrix (FIG. 2A).

15

FIG. 2C. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown are fractured cells taking up DNA as an episomal element, i.e. direct gene transfer *in vivo*.

20

FIG. 2D. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown are fractured repair synthesizing and secreting recombinant proteins encoded by the episomal DNA.

25

FIG. 2E. A schematic model of the cellular and molecular basis of the direct DNA transfer mechanism into osteogenic cells *in vivo*. Shown is the resulting new bone formation.

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FIG. 3A. Achilles' tendon gene transfer is shown as a time course overview at 3 weeks post-surgery.

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FIG. 3B. Achilles' tendon gene transfer is shown as a time course overview at 9 weeks post-surgery.

FIG. 3C. Achilles' tendon gene transfer is shown as
5 a time course overview at 12 weeks post-surgery.

FIG. 3D. Achilles' tendon gene transfer is shown as a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant
10 impregnated with expression plasmid DNA. Note the positive cytoplasmic staining of fibroblastic cells 9 weeks post-surgery.

FIG. 3E. Achilles' tendon gene transfer is shown as
15 a time course immunohistochemistry study. Shown is the microscopy of tendon tissue that received SIS implant alone, without DNA. Note the relative absence of cytoplasmic staining.

FIG. 4. Monitoring of cruciate ligament gene transfer using a substrate utilization assay. Three weeks following the implantation of SIS soaked in a solution of the pSV40 β -gal expression plasmid, tendon tissue was harvested, briefly fixed in 0.5%
25 glutaraldehyde, and then incubated with X-gal according to published methods. Tissues were then embedded in paraffin and sections were cut and stained with H and E. Note the positive (arrows) staining in the cytoplasm of granulation tissues fibroblasts.

30
FIG. 5A. Direct DNA transfer into regenerating bone: β -gal activity. The figure compares β -galactosidase activity in homogenates of osteotomy gap tissue from two Sprague-Dawley rats. In animal #1, the
35 UltraFiber™ implant material was soaked in a solution of pSV40 β -gal DNA, Promega) encoding bacterial β -galactosidase. In animal #2, the implant material was

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soaked in a pure solution of pGL2-Promoter Vector DNA (Promega) encoding insect luciferase. Enzyme activity was determined using substrate assay kits (β -galactosidase and Luciferase Assay Systems, Promega).

- 5 Note that significant β -galactosidase activity was found only in the homogenate prepared from animal #1.

FIG. 5B. Direct DNA transfer into regenerating bone: luciferase activity. The figure compares
10 luciferase activity in aliquots of the homogenates described in FIG. 5A. Luciferase activity was determined using the commercial reagents and protocols (Promega) described in FIG. 5A. Note that significant luciferase activity is found only in the homogenate prepared from
15 animal #2.

FIG. 6A. Osteotomy gene transfer monitored by PTH studies. In this study an expression plasmid coding for a functional 34 amino acid peptide fragment of human
20 parathyroid hormone (PTH1-34) was transferred and expressed in vivo using the GAM technology. The progress of new bone formation in the gap was monitored radiographically for three weeks and the animals were sacrificed. Shown is a radiograph of the osteotomy gap
25 of the control animal that received an antisense hPTH1-34 GAM construct. There was no evidence of radiodense tissue in the gap.

FIG. 6B. Osteotomy gene transfer (FIG. 6A)
30 monitored by PTH studies. Shown is a histological section of osteotomy repair tissue from the same control animal. The section is characterized by the presence of granulation tissue fibroblasts and capillaries.

35 FIG. 6C. Osteotomy gene transfer (FIG. 6A) monitored by PTH studies. Shown is a radiograph of the osteotomy gap that received the sense PTH1-34 GAM

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construct. Note the presence of radiodense tissue in the gap (arrow).

FIG. 6D. Osteotomy gene transfer (FIG. 6A) monitored by PTH studies. Shown is a histological section of osteotomy repair tissue from the same control animal. The section is characterized by the presence of trabecular bone plates that extend into the gap from the surgical margin.

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FIG. 7A. Osteotomy gene transfer BMP-4 studies. Shown is immunohistochemical evidence of BMP-4 transgene expression by granulation tissue fibroblasts near the center of an osteotomy gap three weeks after surgery. Note the positive (arrows) staining of spindled cells. The BMP-4 transgene included an epitope tag (HA epitope, Pharmacia) that facilitated the identification of transgenic BMP-4 molecules. Tissue staining was performed using commercially available polyclonal anti-HA antibodies and standard procedures. Immunostaining was localized only to gap tissues. Control sections included serial sections stained with pre-immune rabbit serum and tissue sections from 13 control osteotomy gaps. In both instances all controls were negative for peroxidase staining of granulation tissue fibroblasts.

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FIG. 7B. Osteotomy gene transfer BMP-4 studies. Shown is the histology of newly formed bone as early as three weeks following gene transfer (FIG. 7A).

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FIG. 8A. Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at six weeks' post surgery. 9 and 16 weeks post-op, are presented in FIG. 8B and FIG. 8C, respectively, to demonstrate the orderly growth of new bone *in situ* over time. This animal, which has been maintained for 23 weeks, has been ambulating normally

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without an external fixator for the past 7 weeks. Similar results have been obtained in a second long term animal (of two) that is now 17 weeks post-op.

5 **FIG. 8B.** Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at nine weeks' post surgery (see FIG. 8A).

10 **FIG. 8C.** Radiographic evidence of bridging new bone formation (arrows) as a consequence of BMP-4 gene transfer and expression at sixteen weeks' post surgery (see FIG. 8A).

15 **FIG. 9A.** The animal shown here is representative of the control group that received an osteotomy plus a collagen sponge without DNA of any type. The animal was maintained for 9 weeks following surgery and then sacrificed. Progress of new bone formation in the gap
20 was monitored radiographically and histologically. Shown is a radiograph of the osteotomy gap at 9 weeks. Note the absence of radiodense tissue in the gap.

FIG. 9B. Shown is a histological section of
25 osteotomy gap tissue from the control animal used in FIG 9A. The section is characterized by the presence of granulation tissue fibroblasts and capillaries.

FIG. 10. PLJ-HPTH1-34 expression construct. A cDNA
30 fragment coding for a prepro-hPTH1-34 peptide was generated by PCR™ (Hendy et al., 1981) and then ligated into a *Bam*HI cloning site in the PLJ retroviral expression vector (Wilson et al., 1992). Several independent clones with the insert in the coding
35 orientation were isolated and characterized.

FIG. 11. Southern analysis of retroviral

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integration in the YZ-15 clone. 10 mg of YZ-15 genomic DNA were digested with KpnI (for which there is a unique site in the vector LTR) and analyzed by Southern blotting. A cDNA fragment that coded for prepro-hPTH1-35 was used as a probe. The positive control for the Southern hybridization conditions was a KpnI digest of genomic DNA from Rat-1 cells infected and selected with the recombinant, replication-defective retrovirus PLJ-hPTH1-84 (Wilson et al., 1992). KpnI digests of DNA were also prepared from two negative controls: native Rat-1 cells and Rat-1 cells infected and selected with BAG ("BAG cells", (Wilson et al., 1992), a replication-defective recombinant retrovirus that encodes β -galactosidase, which is an irrelevant marker gene in these studies. Lane assignments were as follows: 1, PLJ-hPTH1-84 cells; 2 BAG cells; 3, YZ-15; 4, native Rat-1 cells. DNA sizes (kb) are shown at the left of the figure. As expected, a fragment of the predicted size (e.g., 4.3 kb) is seen only in lane 1 (the positive control) and in lane 3 (YZ-15 DNA).

FIG. 12. Northern blot analysis of a transduced Rat-1 clone. Poly-A(+)RNA was prepared from the YZ-15 clone and analyzed by Northern blotting as described (Chen et al., 1993). FIG. 12 contains two panels on a single sheet. Poly-A(+) RNA prepared from PLJ-hPTH1-84 cells, BAG cells, and native Rat-1 cells were used as positive and negative controls. Four probes were applied to a single blot following sequential stripping: hPTH1-34, β -gal, Neo, and β -actin. Lane assignments were as follows: 1, PLJ-hPTH1-84 cells; 2, BAG cells; 3, YZ-15 cells; 4, native Rat-1 cells. As expected, the hPTH1-34 transcript is seen only in lane 1 (positive control) and in lane 3-4; a Neo transcript is seen in lanes 1-3; a β -gal transcript is seen only in lane 2; and β -actin transcripts are seen in lanes 1-4.

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FIG. 13. Northern analysis of poly-A(+) RNA demonstrating expression of the PTH/PTHrP receptor in osteotomy repair tissue.

5 FIG. 14. Overlapping murine cDNA clones representing the LTBP-like (LTBP-3) sequence. A partial representation of restriction sites is shown. N, NcoI; P, PvuII; R, RsaII; B, BamHI; H, HindIII. The numbering system at the bottom assumes that the "A" of the
10 initiator Met codon is nt #1.

FIG. 15A. A schematic showing the structure of the murine fibrillin-1 gene product. Structural domains are shown below the diagram. Symbols designating various
15 structural elements are defined in the legend to FIG. 15B.

FIG. 15B. A schematic showing the structure of the murine LTBP-like (LTBP-3) molecule. Domains #1-5 are
20 denoted below the diagram. Symbols designate the following structural elements: EGF-CB repeats: open rectangles; TGF-bp repeats: open ovals; Fib motif: open circle; TGF-bp-like repeat: patterned oval; cysteine-rich sequences: patterned rectangles; proline/glycine-rich
25 region: thick curved line, domain #2; proline-rich region, thick curved line, domain #3. Note that symbols designating the signal peptide have been deleted for simplicity. Additionally, the schematic assumes that EGF-like and EGF-CB repeats may extend for several amino
30 acids beyond the C_g position.

FIG. 15C. A schematic showing the structure of human LTBP-1. Domains #1-5 are denoted below the diagram. The symbols designating the structural elements
35 are defined in the legend to FIG. 15B.

FIG. 16. Overview of expression of the new LTBP-

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like (LTBP-3) gene during murine development as determined by tissue *in situ* hybridization. FIG. 16 consists of autoradiograms made by direct exposure of tissue sections to film after hybridization with radiolabeled probes. Day 8.5-9.0 sections contained embryos surrounded by intact membranes, uterine tissues, and the placental disk, cut in random planes. Day 13.5 and 16.5 sections contain isolated whole embryos sectioned in the sagittal plane near or about the mid-line. Identical conditions were maintained throughout autoradiography and photography, thereby allowing a comparison of the overall strength of hybridization in all tissue sections. The transcript is expressed in connective tissue, mesenchyme, liver, heart and CNS.

FIG. 17A. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. All photographs in FIG. 17A- FIG. 17D were taken from the same slides used to prepare whole mount sections (after dipping slides in radiographic emulsion). Shown is the neural tube, brightfield image. 1 cm = 20 mm.

FIG. 17B. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. Shown is the neural tube, darkfield image. Note expression by neuroepithelial cells and by surrounding mesenchyme. 1 cm = 20 mm.

FIG. 17C. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse developing tissues. Shown is the heart, brightfield image. The figure demonstrates expression by myocardial and endocardial (arrowheads) cells. 1 cm = 20 mm.

FIG. 17D. Selected microscopic views of mouse LTBP-3 gene expression in day 8.5-9.0 p.c. mouse

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developing tissues. Shown is the heart, darkfield image. The figure demonstrates expression by myocardial and endocardial (arrowheads) cells. Darkfield photomicrographs were taken after exposure of tissues to photographic emulsion for 2 weeks. In this image and the one shown in FIG. 17B, red blood cell and other plasma membranes give a faint white signal that contributes to the background of the experiment. 1 cm = 20 mm.

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FIG. 18A. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. All photographs in FIG. 18A - FIG. 18P were taken from the same slides used to prepare whole mount sections (after dipping slides in radiographic emulsion). Shown is the cartilage model of developing long bone from lower extremity, brightfield image. Expression by chondrocytes and by perichondrial cells is seen in FIG. 18B. 1 cm = 20 mm.

20

FIG. 18B. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the cartilage model of developing long bone from lower extremity, darkfield image. Note expression by chondrocytes and by perichondrial cells. In all darkfield views of FIG. 18, red blood cell and other plasma membranes give a faint white signal that contributes to the background of the experiment. Note the absence of spurious hybridization signal in areas of the slide that lack cellular elements. 1 cm = 20 mm.

30

FIG. 18C. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, brightfield image. 1 cm = 20 mm.

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FIG. 18D. Microscopy of mouse LTBP-3 gene

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expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, brightfield image. 1 cm = 20 mm.

5 **FIG. 18E.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the lung, darkfield image. Note expression by epithelial cells of developing airway and by the surrounding parenchymal cells. 1 cm = 20 mm.

10

FIG. 18F. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the heart, darkfield image. Note continuing expression by myocardial cells. 1 cm = 20 mm.

15

FIG. 18G. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, brightfield image. 1 cm = 20 mm.

20

FIG. 18H. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the intestine, brightfield image. 1 cm = 20 mm.

25

FIG. 18I. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the pancreas, darkfield image. Note expression by acinar epithelial cells. 1 cm = 20 mm.

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FIG. 18J. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is intestine, darkfield image. Note the expression in epithelial and subepithelial cells. 1 cm = 20 mm.

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FIG. 18K. Microscopy of mouse LTBP-3 gene

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expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is kidney, brightfield image. 1 cm = 20 mm.

5 **FIG. 18L.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is skin, brightfield image. 1 cm = 20 mm.

10 **FIG. 18M.** Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is kidney, darkfield image. Note expression by blastemal cells beneath the kidney capsule, epithelial cells of developing nephrons and
15 tubules, and the interstitial mesenchyme. 1 cm = 20 mm.

FIG. 18N. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing tissues. Shown is the skin, darkfield image. Note the
20 expression by epidermal, adnexal and dermal cells of developing skin. 1 cm = 20 mm.

FIG. 18O. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing
25 tissues. Shown is the retina, brightfield image. 1 cm = 20 mm.

FIG. 18P. Microscopy of mouse LTBP-3 gene expression in day 13.5 and day 16 p.c. mouse developing
30 tissues. Shown is the retina, darkfield image. Note expression by retinal epithelial cells and by adjacent connective tissue cells. 1 cm = 20 mm.

FIG. 19. Time-dependent expression of the LTBP-3
35 gene by MC3T3-E1 cells. mRNA preparation and Northern blotting were performed as described in Example XIV. Equal aliquots of total RNA as determined by UV

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spectroscopy were loaded in each lane of the Northern gel. As demonstrated by UV spectroscopy were loaded in each lane of the Northern gel. As demonstrated by methylene blue staining (Sambrook et al., 1989), equal
5 amounts of RNA were transferred to the nylon membrane. The results demonstrate a clear, strong peak in LTBP-3 gene expression by 14 days in culture. Weaker signals denoting LTBP-3 gene expression also can be observed after 5 days and 28 days in culture.

10

FIG. 20. Antisera #274 specifically binds LTBP-3 epitopes. Transfection of 293T cells with a full length mouse LTBP-3 expression plasmid followed by radiolabeling, preparation of medium sample,
15 immunoprecipitation, and 4-18% gradient SDS-PAGE were performed as described in Example XIV. The figure presents a SDS-PAGE autoradiogram of medium samples following a 2 day exposure to film. Lane assignments are as follows: Lane 1, radiolabeled 293T medium (prior to transfection) immunoprecipitated with preimmune serum;
20 Land 2, radiolabeled 293T medium (prior to transfection) immunoprecipitated with antibody #274; Lane 3, radiolabeled 393T medium (following transfection and preincubation with 10 μ g of LTBP-3 synthetic peptide cocktail) immunoprecipitated with antibody #274; and Lane
25 4, radiolabeled 293T medium (following transfection) immunoprecipitated with antibody #274. As indicated by the bar, the full length LTBP-3 molecule migrated at 180-190 kDa.

30

FIG. 21. Co-immunoprecipitation of LTBP-3 and TGR- β 1 produced by MC3T3-E1 cells. Aliquots ($\sim 10^6$ incorporated CPM) of radiolabeled media produced by MC3T3-E1 cells after 7 days in culture were
35 immunoprecipitated as described in Example XIV. Bars indicate the position of cold molecular weight standards used to estimate molecular weight (Rainbow mix,

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Amersham). Immunoprecipitates were separated using 4%-18% gradient SDS-PAGE and reducing conditions. The figure shows a negative control lane 1 consisting of MC3T3-E1 medium immunoprecipitated with anti-LTBP-3 antibody #274. Western blotting was performed using the lower portion of the gradient gel and a commercially available antibody to TGF- β 1 (Santa Cruz Biotechnology, Inc.). Antibody staining was detected using commercially available reagents and protocols (ECL Western Blotting Reagent, Amersham). MC3T3-E1 medium was immunoprecipitated with anti-LTBP-2 antibody #274.

FIG. 22A. Radiographic analysis of the type II collagen osteotomy gap three weeks after surgery.

FIG. 22B. Radiographic analysis of the type I collagen osteotomy gap three weeks after surgery.

FIG. 22C. Histologic analysis of the type II collagen osteotomy shown in FIG. 22A.

FIG. 23A. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Positive (arrows) β -gal cytoplasmic staining is observed in the fracture repair cells.

FIG. 23B. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Serial section negative control stained with the vehicle of the β -gal antibody plus a cocktail of non-specific rabbit IgG antibodies.

FIG. 23C. Adenovirus-mediated gene transfer into bone repair/regeneration cells *in vivo*. Osteotomy site was filled with a fibrous collagen implant material soaked in a solution of the replication-defective recombinant adenovirus AdRSV β -gal ($\sim 10^{11}$ plaque forming

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units/ml). Note the positive (arrow) β -gal nuclear staining of chondrocytes within the osteotomy site, as demonstrated by immunohistochemistry using a specific anti- β -gal antibody.

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FIG. 24. The murine BMP-4 amino acid sequence, SEQ ID NO:1. The HA epitope is shown in bold at the extreme carboxy terminus of the sequence.

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FIG. 25. DNA sequence of the murine LTBP-3 gene (SEQ ID NO:2).

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FIG. 26. Amino acid sequence of the murine LTBP-3 gene product (SEQ ID NO:3).

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FIG. 27. DNA sequence of the murine LTBP-2 gene (SEQ ID NO:17).

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FIG. 28. Amino acid sequence of the murine LTBP-2 gene product (SEQ ID NO:18).

DESCRIPTION OF THE PREFERRED EMBODIMENT

25 1. Applications of Bone Repair Technology to Human Treatment

The following is a brief discussion of four human conditions to exemplify the variety of diseases and disorders that would benefit from the development of new technology to improve bone repair and healing processes. In addition to the following, several other conditions, such as, for example, vitamin D deficiency; wound healing in general; soft skeletal tissue repair; and cartilage and tendon repair and regeneration, may also benefit from technology concerning the stimulation of bone progenitor cells.

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The first example is the otherwise healthy individual who suffers a fracture. Often, clinical bone fracture is treated by casting to alleviate pain and allow natural repair mechanisms to repair the wound.

5 While there has been progress in the treatment of fracture in recent times, even without considering the various complications that may arise in treating fractured bones, any new procedures to increase bone healing in normal circumstances would represent a great
10 advance.

A second example which may benefit from new treatment methods is osteogenesis imperfecta (OI). OI encompasses various inherited connective tissue diseases
15 that involve bone and soft connective tissue fragility in humans (Byers and Steiner, 1992; Prockop, 1990). About one child per 5,000-20,000 born is affected with OI and the disease is associated with significant morbidity throughout life. A certain number of deaths also occur,
20 resulting in part from the high propensity for bone fracture and the deformation of abnormal bone after fracture repair (OI types II-IV; Bonadio and Goldstein, 1993). The relevant issue here is quality of life; clearly, the lives of affected individuals would be
25 improved by the development of new therapies designed to stimulate and strengthen the fracture repair process.

OI type I is a mild disorder characterized by bone fracture without deformity, blue sclerae, normal or near
30 normal stature, and autosomal dominant inheritance (Bonadio and Goldstein, 1993). Osteopenia is associated with an increased rate of lone bone fracture upon ambulation (the fracture frequency decreases dramatically at puberty and during young adult life, but increases
35 once again in late middle age). Hearing loss, which often begins in the second or third decade, is a feature of this disease in about half the families and can

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progress despite the general decline in fracture frequency. Dentinogenesis imperfecta is observed in a subset of individuals.

5 In contrast, OI types II-VI represent a spectrum of more severe disorders associated with a shortened life-span. OI type II, the perinatal lethal form, is characterized by short stature, a soft calvarium, blue sclerae, fragile skin, a small chest, floppy appearing
10 lower extremities (due to external rotation and abduction of the femurs), fragile tendons and ligaments, bone fracture with severe deformity, and death in the perinatal period due to respiratory insufficiency. Radiographic signs of bone weakness include compression
15 of the femurs, bowing of the tibiae, broad and beaded ribs, and calvarial thinning.

 OI type III is characterized by short stature, a triangular facies, severe scoliosis, and bone fracture
20 with moderate deformity. Scoliosis can lead to emphysema and a shortened life-span due to respiratory insufficiency. OI type IV is characterized by normal sclerae, bone fracture with mild to moderate deformity, tooth defects, and a natural history that essentially is
25 intermediate between OI type II and OI type I.

 More than 200 OI mutations have been characterized since 1989 (reviewed in Byers and Steiner, 1992; Prockop, 1990). The vast majority occur in the COL1A1 and COL1A2
30 genes of type I collagen. Most cases of OI type I appear to result from heterozygous mutations in the COL1A1 gene that decrease collagen production but do not alter primary structure, i.e., heterozygous null mutations affecting COL1A1 expression. Most cases of OI types II-
35 IV result from heterozygous mutations in the COL1A1 and COL1A2 genes that alter the structure of collagen.

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A third important example is osteoporosis. The term osteoporosis refers to a heterogeneous group of disorders characterized by decreased bone mass and fractures. An estimated 20-25 million people are at increased risk for fracture because of site-specific bone loss. Risk factors for osteoporosis include increasing age, gender (more females), low bone mass, early menopause, race (Caucasians), low calcium intake, reduced physical activity, genetic factors, environmental factors (including cigarette smoking and abuse of alcohol or caffeine), and deficiencies in neuromuscular control that create a propensity to fall.

More than a million fractures in the USA each year can be attributed to osteoporosis, and in 1986 alone the treatment of osteoporosis cost an estimated 7-10 billion health care dollars. Demographic trends (i.e., the gradually increasing age of the US population) suggest that these costs may increase 2-3 fold by the year 2020 if a safe and effective treatment is not found. Clearly, osteoporosis is a significant health care problem.

Clinically, osteoporosis is segregated into type I and type II. Type I osteoporosis occurs predominantly in middle aged women and is associated with estrogen loss at the menopause, while osteoporosis type II is associated with advancing age. Much of the morbidity and mortality associated with osteoporosis results from immobilization of elderly patients following fracture.

Current therapies for osteoporosis patients focus on fracture prevention, not fracture repair. This remains an important consideration because of the literature, which clearly states that significant morbidity and mortality are associated with prolonged bed rest in the elderly, particularly those who have suffered hip fractures. Complications of bed rest include blood clots

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and pneumonia. These complications are recognized and measures are usually taken to avoid them, but these measures hardly represent the best approach to therapy. Thus, the osteoporotic patient population would benefit from new therapies designed to strengthen bone and speed up the fracture repair process, thereby getting these people on their feet before the complications arise.

A fourth example is related to bone reconstruction and, specifically, the ability to reconstruct defects in bone tissue that result from traumatic injury; cancer or cancer surgery; birth defect; a developmental error or heritable disorder; or aging. There is a significant orthopaedic need for more stable total joint implants, and cranial and facial bone are particular targets for this type of reconstructive need. The availability of new implant materials, e.g., titanium, has permitted the repair of relatively large defects. Titanium implants provide excellent temporary stability across bony defects. However, experience has shown that a lack of viable bone bridging the defect can result in exposure of the appliance, infection, structural instability and, ultimately, failure to repair the defect.

Autologous bone grafts are another possible reconstructive modality, but they have several demonstrated disadvantages in that they must be harvested from a donor site such as iliac crest or rib, they usually provide insufficient bone to completely fill the defect, and the bone that does form is sometimes prone to infection and resorption. Partially purified xenogeneic preparations are not practical for clinical use because microgram quantities are purified from kilograms of bovine bone, making large scale commercial production both costly and impractical. Allografts and demineralized bone preparations are therefore often employed.

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Microsurgical transfers of free bone grafts with attached soft tissue and blood vessels can close bony defects with an immediate source of blood supply to the graft. However, these techniques are time consuming, have been shown to produce a great deal of morbidity, and can only be used by specially trained individuals. Furthermore, the bone implant is often limited in quantity and is not readily contoured. In the mandible, for example, the majority of patients cannot wear dental appliances using presently accepted techniques (even after continuity is established), and thus gain little improvement in the ability to masticate. Toriumi et al., have written that, "reconstructive surgeons should have at their disposal a bone substitute that would be reliable, biocompatible, easy to use, and long lasting and that would restore mandibular continuity with little associated morbidity."

In connection with bone reconstruction, specific problem areas for improvement are those concerned with treating large defects, such as created by trauma, birth defects, or particularly, following tumor resection. The success of orthopaedic implants, interfaces and artificial joints could conceivably be improved if the surface of the implant, or a functional part of an implant, were to be coated with a bone stimulatory agent. The surface of implants could be coated with one or more appropriate materials in order to promote a more effective interaction with the biological site surrounding the implant and, ideally, to promote tissue repair.

2. Bone Repair

Bone tissue is known to have the capacity for repair and regeneration and there is a certain understanding of the cellular and molecular basis of these processes. The

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initiation of new bone formation involves the commitment, clonal expansion, and differentiation of progenitor cells. Once initiated, bone formation is promoted by a variety of polypeptide growth factors. Newly formed bone is then maintained by a series of local and systemic growth and differentiation factors.

The concept of specific bone growth-promoting agents is derived from the work of Huggins and Urist. Huggins et al., 1936, demonstrated that autologous transplantation of canine incisor tooth to skeletal muscle resulted in local new bone formation (Huggins et al., 1936). Urist and colleagues reported that demineralized lyophilized bone segments induced bone formation (Urist, 1965; Urist et al., 1983), a process that involved macrophage chemotaxis; the recruitment of progenitor cells; the formation of granulation tissue, cartilage, and bone; bone remodeling; and marrow differentiation. The initiation of cartilage and bone formation in an extraskeletal site, a process referred to as osteoinduction, has permitted the unequivocal identification of initiators of bone morphogenesis (Urist, 1965; Urist et al., 1983; Sampath et al., 1984; Wang et al., 1990; Cunningham et al., 1992).

Significant progress has now been made in characterizing the biological agents elaborated by active bone tissue during growth and natural bone healing. Demineralized bone matrix is highly insoluble; Sampath and Reddi (1981) showed that only 3% of the proteins can be extracted using strong combinations of denaturants and detergents. They also showed that the unfractionated demineralized bone extract will initiate bone morphogenesis, a critical observation that led to the purification of "osteoinductive" molecules. Families of proteinaceous osteoinductive factors have now been purified and characterized. They have been variously

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referred to in the literature as bone morphogenetic or morphogenic proteins (BMPs), osteogenic bone inductive proteins or osteogenic proteins (OPs).

5 3. Bone Repair and Bone Morphogenetic Proteins (BMPs)

Following their initial purification, several bone morphogenetic protein genes have now been cloned using molecular techniques (Wozney et al., 1988; Rosen et al.,
10 1989; summarized in Alper, 1994). This work has established BMPs as members of the transforming growth factor- β (TGF- β) superfamily based on DNA sequence homologies. Other TGF molecules have also been shown to participate in new bone formation, and TGF- β is regarded
15 as a complex multifunctional regulator of osteoblast function (Centrella et al., 1988; Carrington et al., 1988; Seitz et al., 1992). Indeed, the family of transforming growth factors (TGF- β 1, TGF- β 2, and TGF- β 3) has been proposed as potentially useful in the treatment
20 of bone disease (U.S. Patent 5,125,978, incorporated herein by reference).

The cloning of distinct BMP genes has led to the designation of individual BMP genes and proteins as BMP-1
25 through BMP-8. BMPs 2-8 are generally thought to be osteogenic (BMP-1 may be a more generalized morphogen; Shimell et al., 1991). BMP-3 is also called osteogenin (Luyten et al., 1989) and BMP-7 is also called OP-1 (Ozkaynak et al., 1990). TGFs and BMPs each act on cells
30 via complex, tissue-specific interactions with families of cell surface receptors (Roberts and Sporn, 1989; Paralkar et al., 1991).

Several BMP (or OP) nucleotide sequences and
35 vectors, cultured host cells and polypeptides have been described in the patent literature. For example, U.S. Patents, 4,877,864, 4,968,590 and 5,108,753 all concern

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osteogenic factors. More specifically, BMP-1 is disclosed in U.S. Patent 5,108,922; BMP-2 species, including MBP-2A and BMP-2B, are disclosed in U.S. Patents 5,166,058, 5,013,649, and 5,013,649; BMP-3 in 5,116,738; BMP-5 in 5,106,748; BMP-6 in 5,187,076; and BMP-7 in 5,108,753 and 5,141,905; all incorporated herein by reference. Various BMP clones and their activities are particularly described by Wozney et al., (1988; incorporated herein by reference). DNA sequences encoding the osteogenic proteins designated OP-1, COP-5 and COP-7 are also disclosed in U.S. Patent 5,011,691. Although the BMP terminology is widely used, it may prove to be the case that there is an OP counterpart term for every individual BMP (Alper, 1994).

15

4. Bone Repair and Growth Factors and Cytokines

Transforming growth factors (TGFs) have a central role in regulating tissue healing by affecting cell proliferation, gene expression, and matrix protein synthesis (Roberts and Sporn, 1989). While not necessarily a direct effect, Bolander and colleagues have provided evidence that TGF- β 1 and TGF- β 2 can initiate both chondrogenesis and osteogenesis (Joyce et al., 1990; Izumi et al., 1992; Jingushi et al., 1992). In these studies new cartilage and bone formation appeared to be dose dependent (i.e., dependent on the local growth factor concentration). The data also suggested that TGF- β 1 and TGF- β 2 stimulated cell differentiation by a similar mechanism, even though they differed in terms of the ultimate amount of new cartilage and bone that was formed.

Other growth factors/hormones besides TGF and BMP may influence new bone formation following fracture. Bolander and colleagues injected recombinant acidic fibroblast growth factor into a rat fracture site

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(Jingushi et al., 1990). The major effect of multiple high doses (1.0 mg/50 ml) was a significant increase in cartilage tissue in the fracture gap, while lower doses had no effect. These investigators also used the reverse transcriptase-polymerase chain reaction (PCR™) technique to demonstrate expression of estrogen receptor transcripts in callus tissue (Boden et al., 1989). These results suggested a role for estrogen in normal fracture repair.

Horowitz and colleagues have shown that activated osteoblasts will synthesize the cytokine, macrophage colony stimulating factor (Horowitz et al., 1989). The osteotropic agents used in this study included lipopolysaccharide, PTH1-84, PTH1-34, vitamin D and all-trans retinoic acid. This observation has led to the suggestion that osteoblast activation following fracture may lead to the production of cytokines that regulate both hematopoiesis and new bone formation. Various other proteins and polypeptides that have been found to be expressed at high levels in osteogenic cells, such as, e.g., the polypeptide designated Vgr-1 (Lyons et al., 1989), also have potential for use in connection with the present invention.

5. Bone Repair and Calcium Regulating Hormones

Calcium regulating hormones such as parathyroid hormone (PTH) participate in new bone formation and bone remodeling (Raisz and Kream, 1983). PTH is an 84 amino acid calcium-regulating hormone whose principle function is to raise the Ca^{+2} concentration in plasma and extracellular fluid. Studies with the native hormone and with synthetic peptides have demonstrated that the amino-terminus of the molecule (aa 1-34) contains the structural requirements for biological activity (Tregear et al., 1973; Hermann-Erlee et al., 1976; Riond, 1993).

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PTH functions by binding to a specific cell surface receptor that belongs to the G protein-coupled receptor superfamily (Silve et al., 1982; Rizzoli et al., 1983; Juppner et al., 1991).

5

Using a retroviral approach, a human full-length PTH gene construct has been introduced into cultured rat fibroblasts to create recombinant PTH-secreting cells. These cells were then transplanted into syngeneic rat recipients that were observed to develop hypercalcemia mediated by the increased serum concentrations of PTH (Wilson et al., 1992). The object of these studies was to create an animal model of primary hyperparathyroidism.

15

PTH has a dual effect on new bone formation, a somewhat confusing aspect of hormone function despite intensive investigation. PTH has been shown to be a potent direct inhibitor of type I collagen production by osteoblasts (Kream et al., 1993). Intact PTH was also shown to stimulate bone resorption in organ culture over 30 years ago, and the hormone is known to increase the number and activity of osteoclasts. Recent studies by Gay and colleagues have demonstrated binding of [¹²⁵I]PTH(1-84) to osteoclasts in tissue sections and that osteoclasts bind intact PTH in a manner that is both saturable and time- and temperature dependent (Agarwala and Gay, 1992). While these properties are consistent with the presence of PTH/PTHrP receptors on the osteoclast cell surface, this hypothesis is still considered controversial. A more accepted view, perhaps, is that osteoclast activation occurs via an osteoblast signaling mechanism.

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On the other hand, osteosclerosis may occur in human patients with primary hyperparathyroidism (Seyle, 1932). It is well known that individuals with hyperparathyroidism do not inexorably lose bone mass, but

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eventually achieve a new bone remodeling steady state after an initial period of net bone loss. Chronic, low dose administration of the amino-terminal fragment of PTH (aa 1-34) also can induce new bone formation according to a time- and dose-dependent schedule (Seyle, 1932; Parsons and Reit, 1974).

Human PTH1-34 has recently been shown to: stimulate DNA synthesis in chick osteoblasts and chondrocytes in culture (van der Plas, 1985; Schluter et al., 1989; Somjen et al., 1990); increase bone cell number *in vivo* (Malluche et al., 1986); enhance the *in vitro* growth of chick embryonic cartilage and bone (Kawashima, 1980; Burch and Lebovitz, 1983; Lewinson and Silbermann, 1986; Endo et al., 1980; Klein-Nulend et al., 1990); enhance surface bone formation (both cortical and trabecular bone) in normal and osteogenic animals and in humans with osteoporosis (Reeve et al., 1976; Reeve et al., 1980; Tam et al., 1982; Hefti et al., 1982; Podbesek et al., 1983; Stevenson and Parsons, 1983; Slovik et al., 1986; Gunness-Hey and Hock, 1984; Tada et al., 1988; Spencer et al., 1989; Hock and Fonseca, 1990; Liu and Kalu, 1990; Hock and Gera, 1992; Mitlak et al., 1992; Ejersted et al., 1993); and delay and reverse the catabolic effects of estrogen deprivation on bone mass (Hock et al., 1988; Hori et al., 1988; Gunness-Hey and Hock, 1989; Liu et al., 1991). Evidence of synergistic interactions between hPTH-1-34 and other anabolic molecules has been presented, including insulin-like growth factor, BMP-2, growth hormone, vitamin D, and TGF- β (Slovik et al., 1986; Spencer et al., 1989; Mitlak et al., 1992; Canalis et al., 1989; Linkhart and Mohan, 1989; Seitz et al., 1992; Vukicevic et al., 1989).

Anecdotal observation has shown that serum PTH levels may be elevated following bone fracture (Meller et al., 1984; Johnston et al., 1985; Compston et al., 1989;

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Hardy et al., 1993), but the significance of this observation is not understood. There are apparently no reports in the literature concerning attempts to localize either PTH or the PTH/PTHrP receptor *in situ* in human fracture sites or in experimental models. Furthermore, no attempt has been made to augment bone repair by the exogenous addition of PTH peptides. Although hPTH1-34 is known to function as an anabolic agent for bone, prior to the present invention, much remained to be learned about the role (if any) of PTH during bone regeneration and repair.

6. Protein Administration and Bone Repair

Several studies have been conducted in which preparations of protein growth factors, including BMPs, have been administered to animals in an effort to stimulate bone growth. The results of four such exemplary studies are described below.

Toriumi et al., studied the effect of recombinant BMP-2 on the repair of surgically created defects in the mandible of adult dogs (Toriumi et al., 1991). Twenty-six adult hounds were segregated into three groups following the creation of a 3 cm full thickness mandibular defect: 12 animals received test implants composed of inactive dog bone matrix carrier and human BMP-2, 10 animals received control implants composed of carrier without BMP-2, and BMP-4 animals received no implant. The dogs were euthanized at 2.5-6 months, and the reconstructed segments were analyzed by radiography, histology, histomorphometry, and biomechanical testing. Animals that received test implants were euthanized after 2.5 months because of the presence of well mineralized bone bridging the defect. The new bone allowed these animals to chew a solid diet, and the average bending strength of reconstructed mandibles was 27% of normal

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('normal' in this case represents the unoperated, contralateral hemimandible). In contrast, the implants in the other two groups were non-functional even after 6 months and showed minimal bone formation.

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Yasko et al., published a related study in which the effect of BMP-2 on the repair of segmental defects in the rat femur was examined (Yasko et al., 1992). The study design included a group that received a dose of 1.4 mg of BMP-2, another group that received 11.0 mg of BMP-2, and a control group that received carrier matrix alone. Endochondral bone formation was observed in both groups of animals that received BMP-2. As demonstrated by radiography, histology, and whole bone (torsion) tests of mechanical integrity, the larger dose resulted in functional repair of the 5-mm defect beginning 4.5 weeks after surgery. The lower dose resulted in radiographic and histological evidence of new bone formation, but functional union was not observed even after 9 weeks post surgery. There was also no evidence of bone formation in control animals at this time.

Chen et al., showed that a single application of 25-100 mg of recombinant TGF- β 1 adjacent to cartilage induced endochondral bone formation in the rabbit ear full thickness skin wounds (Chen et al., 1991). Bone formation began 21 days following the creation of the wound and reached a peak at day 42, as demonstrated by morphological methods. Active bone remodeling was observed beyond this point.

In a related study, Beck et al., demonstrated that a single application of TGF- β 1 in a 3% methylcellulose gel was able to repair surgically induced large skull defects that otherwise heal by fibrous connective tissue and never form bone (Beck et al., 1991). Bony closure was achieved within 28 days of the application of 200 mg of

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TGF- β 1 and the rate of healing was shown to be dose dependent.

Studies such as those described above have thus
5 established that exogenous growth factors can be used to
stimulate new bone formation/repair/regeneration in vivo.
Certain U.S. Patents also concern methods for treating
bone defects or inducing bone formation. For example,
U.S. Patent 4,877,864 relates to the administration of a
10 therapeutic composition of bone inductive protein to
treat cartilage and/or bone defects; U.S. Patent
5,108,753 concerns the use of a device containing a pure
osteogenic protein to induce endochondral bone formation
and for use in periodontal, dental or craniofacial
15 reconstructive procedures.

However, nowhere in this extensive literature does
there appear to be any suggestion that osteogenic genes
themselves may be applied to an animal in order to
20 promote bone repair or regeneration. Indeed, even
throughout the patent literature that concerns genes
encoding various bone stimulatory factors and their in
vitro expression in host cells to produce recombinant
proteins, there seems to be no mention of the possibility
25 of using nucleic acid transfer in an effort to express an
osteogenic gene in bone progenitor cells in vivo or to
promote new bone formation in an animal or human subject.

7. Biocompatible Matrices for use in Bone Repair

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There is a considerable amount of work that has been
directed to the development of biocompatible matrices for
use in medical implants, including those specifically for
bone implantation work. In context of the present
35 invention, a matrix may be employed in association with
the gene or DNA coding region encoding the osteotropic
polypeptide in order to easily deliver the gene to the

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site of bone damage. Such matrices may be formed from a variety of materials presently in use for implanted medical applications.

5 In certain cases, the matrix may also act as a "biofiller" to provide a structure for the developing bone and cartilage. However, the formation of such a scaffolding structure is not a primary requirement, rather, the main requirements of the matrix are to be
10 biocompatible and to be capable of delivering a nucleic acid segment to a bone cell or bone tissue site.

Matrices that may be used in certain embodiments include non-biodegradable and chemically defined
15 matrices, such as sintered hydroxyapatite, bioglass, aluminates, and other ceramics. The bioceramics may be altered in composition, such as in calcium-aluminate-phosphate; and they may be processed to modify particular physical and chemical characteristics, such as pore size,
20 particle size, particle shape, and biodegradability. Certain polymeric matrices may also be employed if desired, these include acrylic ester polymers and lactic acid polymers, as disclosed in U.S. Patents 4,526,909, and 4,563,489, respectively, each incorporated herein by
25 reference. Particular examples of useful polymers are those of orthoesters, anhydrides, propylene-cofumarates, or a polymer of one or more α -hydroxy carboxylic acid monomers, e.g., α -hydroxy acetic acid (glycolic acid) and/or α -hydroxy propionic acid (lactic acid).

30 Some of the preferred matrices for use in present purposes are those that are capable of being resorbed into the body. Potential biodegradable matrices for use in bone gene transfer include, for example, PLGA block
35 copolymers, biodegradable and chemically defined calcium sulfate, tricalciumphosphate, hydroxyapatite, and polyanhydrides. Furthermore, biomatrices comprised of

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pure proteins and/or extracellular matrix components may be employed.

The inventors have shown the use of bone or dermal collagenous materials as matrices, as may be prepared from various commercially-available lyophilized collagen preparations, such as those from bovine or rat skin, as well as PLGA block copolymers. Collagen matrices may also be formulated as described in U.S. Patent 4,394,370, incorporated herein by reference, which concerns the use of collagenous matrices as delivery vehicles for osteogenic protein. UltraFiber™, as may be obtained from Norian Corp. (Mountain View, CA), is a preferred matrix. Preferred matrices are those formulated with type II collagen, and most preferably, recombinant type II collagen and mineralized type II collagen.

Further suitable matrices may also be prepared from combinations of materials, such as PLGA block copolymers, which allow for sustained release; hydroxyapatite; or collagen and tricalciumphosphate. Although sufficient sequestration and subsequent delivery of an osteotropic gene is in no way a limitation of the present invention, should it be desired, a porous matrix and gene combination may also be administered to the bone tissue site in combination with an autologous blood clot. The basis for this is that blood clots have previously been employed to increase sequestration of osteogenic proteins for use in bone treatment (U.S. Patent 5,171,579, incorporated herein by reference) and their use in connection with the present invention is by no means excluded (they may even attract growth factors for cytokines).

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8. Collagen

Although not previously proposed for use with a nucleic acid molecule, the use of collagen as a pharmaceutical delivery vehicle has been described. The biocompatibility of collagen matrices is well known in the art. U.S. Patents 5,206,028, 5,128,136, 5,081,106, 4,585,797, 4,390,519, and 5,197,977 (all incorporated herein by reference) describe the biocompatibility of collagen-containing matrices in the treatment of skin lesions, use as a wound dressing, and as a means of controlling bleeding. In light of these documents, therefore, there is no question concerning the suitability of applying a collagen preparation to a tissue site of an animal.

U.S. Patent 5,197,977 describes the preparation of a collagen-impregnated vascular graft including drug materials complexed with the collagen to be released slowly from the graft following implant. U.S. Patent 4,538,603 is directed to an occlusive dressing useful for treating skin lesions and a granular material capable of interacting with wound exudate. U.S. Patent 5,162,430 describes a pharmaceutically acceptable, non-immunogenic composition comprising a telopeptide collagen chemically conjugated to a synthetic hydrophilic polymer.

Further documents that one of skill in the art may find useful include U. S. Patents 4,837,285, 4,703,108, 4,409,332, and 4,347,234, each incorporated herein by reference. These references describe the uses of collagen as a non-immunogenic, biodegradable, and bioresorbable binding agent.

The inventors contemplate that collagen from many sources will be useful in the present invention. Particularly useful are the amino acid sequences of type

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II collagen. Examples of type II collagen are well known in the art. For example, the amino acid sequences of human (Lee et al., 1989), rat (Michaelson et al., 1994), and murine (Ortman et al., 1994) have been determined
5 (SEQ ID NO:10, SEQ ID NO:12, and SEQ ID NO:14, respectively).

Although not previously known to be capable of stimulating bone progenitor cells itself, type II
10 collagen is herein surprisingly shown to possess this property, which thus gives rise to new possibilities for clinical uses.

9. Nucleic Acid Delivery

15 The transfer of nucleic acids to mammalian cells has been proposed a method for treating certain diseases or disorders. Nucleic acid transfer or delivery is often referred to as "gene therapy". Initial efforts toward
20 postnatal (somatic) gene therapy relied on indirect means of introducing genes into tissues, e.g., target cells were removed from the body, infected with viral vectors carrying recombinant genes, and implanted into the body. These type of techniques are generally referred to as ex
25 vivo treatment protocols. Direct in vivo gene transfer has recently been achieved with formulations of DNA trapped in liposomes (Ledley et al., 1987); or in proteoliposomes that contain viral envelope receptor proteins (Nicolau et al., 1983); calcium phosphate-
30 coprecipitated DNA (Benvenisty and Reshef, 1986); and DNA coupled to a polylysine-glycoprotein carrier complex (Wu and Wu, 1988). The use of recombinant replication-defective viral vectors to infect target cells in vivo has also been described (e.g., Seeger et al., 1984).

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In recent years, Wolff et al., demonstrated that direct injection of purified preparations of DNA and RNA

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into murine skeletal muscle resulted in significant reporter gene expression (Wolff et al., 1990). This was an unexpected finding, and the mechanism of gene transfer could not be defined. The authors speculated that muscle cells may be particularly suited to take up and express polynucleotides *in vivo* or that damage associated with DNA injection may allow transfection to occur.

Wolff et al., suggested several potential applications of the direct injection method, including (a) the treatment of heritable disorders of muscle, (b) the modification of non-muscle disorders through muscle tissue expression of therapeutic transgenes, (c) vaccine development, and (d) a reversible type of gene transfer, in which DNA is administered much like a conventional pharmaceutical treatment. In an elegant study Liu and coworkers recently showed that the direct injection method can be successfully applied to the problem of influenza vaccine development (Ulmer et al., 1993).

The use of gene transfer to synoviocytes as a means of treating arthritis has also been discussed (Bandara et al., 1992; Roessler et al., 1993). The protocols considered have included both the *ex vivo* treatment of isolated synoviocytes and their re-introduction into the animal and also direct gene transfer in which suitable vectors are injected into the joint. The transfer of marker genes into synoviocytes has already been demonstrated using both retroviral and adenoviral technology (Bandara et al., 1992; Roessler et al., 1993).

Despite the exclusive emphasis on protein treatment by those working in the field of new bone growth, the present inventors saw that there was great potential for using nucleic acids themselves to promote bone regeneration/repair *in vivo*. This provides for a more sophisticated type of pharmaceutical delivery. In

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addition to the ease and cost of preparing DNA, it was also reasoned that using DNA transfer rather than peptide transfer would provide many further advantages. For example, DNA transfer allows for the expression or over-
5 expression of integral membrane receptors on the surface of bone regeneration/repair cells, whereas this cannot be done using peptide transfer because the latter (*a priori*) is an extracellular manipulation. Importantly, DNA transfer also allows for the expression of polypeptides
10 modified in a site-directed fashion with the very minimum of additional work (*i.e.*, straightforward molecular biological manipulation without protein purification) as well as sustained release of therapies delivered by an injectable route.

15 The advantages of using DNA are also manifold regarding the development of pharmaceutical products and effective means of delivery. Here, important advantages include the ability to prepare injectable formulations,
20 especially those compositions that exhibit reversible thermal gelation, and the opportunity to combine such injectables with imaging technology during delivery. "Sustained release" is also an important advantage of using DNA, in that the exogenously added DNA continues to
25 direct the production of a protein product following incorporation into a cell. The use of certain matrix-DNA compositions also allows for a more typical "sustained release" phenomenon in that the operative release of DNA from the matrix admixture can also be manipulated.

30 The inventors contemplated that both naked DNA and viral-mediate DNA could be employed in an effort to transfer genes to bone progenitor cells. In beginning to study this, the most appropriate animal model had to be
35 employed, that is, one in which the possibilities of using nucleic acids to promote bone repair could be adequately tested in controlled studies.

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10. Osteotomy Model

Prior to the present invention, three model systems were available for study in this area, including Mov13 mice, an animal model of OI. Unfortunately, each of the models suffers from significant drawbacks. With the Mov13 mice, first, these mice typically die in young adulthood because of retrovirus-induced leukemia (Schnieke et al., 1983); second, gene transfer studies in Mov13 mice conducted between postnatal weeks 8-16 (i.e., prior to the development of leukemia) may be complicated by a natural adaptation in which a significant amount of new bone is deposited on the periosteal surface (Bonadio et al., 1993); and third, an osteotropic gene transferred into an osteotomy site may synergize with the active retrovirus and make it even more virulent.

Another system is the *in vivo* bone fracture model created by Einhorn and colleagues (Bonnarens and Einhorn, 1984). However, this model is a closed system that would not easily permit initial studies of gene transfer *in vivo*. The organ culture model developed by Bolander and colleagues (Joyce et al., 1990) was also available, but again, this model is not suitable for studying gene transfer *in vivo*. Due to the unsuitability of the above models for studying the effects of gene transfer on bone repair and regeneration, the inventors employed a rat osteotomy system, as described below.

The important features of the rat osteotomy model are as follows: under general anesthesia, four 1.2 mm diameter pins are screwed into the femoral diaphysis of normal adult Sprague-Dawley rats. A surgical template ensures parallel placement of the pins. An external fixator is then secured on the pins, and a 2 mm, or 5 mm, segmental defect is created in the central diaphysis with a Hall micro 100 oscillating saw. A biodegradable

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implant material, soaked in a solution of plasmid DNA, other genetic construct or recombinant virus preparation, is then placed in the intramedullary canal and the defect is closed (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

New bone formation can be detected as early as three weeks later in the 2 mm gap, although up to 9 weeks is generally allowed for new bone formation to occur. The fixator provided the necessary stability, and there were no limitations on animal ambulation. The surgical protocol has been successfully performed on 21/21 animals to date. None of these animals have died. Assays of new bone formation are performed after sacrifice, except plain film radiography, which is performed weekly from the time of surgery to sacrifice.

Previous studies in Sprague-Dawley rats have shown that the 5 mm osteotomy gap will heal as a fibrous non-union, whereas a gap of less than 3 mm, (such as the 2 mm gap routinely employed in the studies described herein) will heal by primary bone formation. Studies using the 5 mm gap thus allow a determination of whether transgene expression can stimulate new bone formation when fibrous tissue healing normally is expected. On the other hand, studies with the 2 mm gap allow a determination of whether transgene expression can speed up natural primary bone healing. Controls were also performed in which animals received no DNA (FIG. 9A and FIG. 9B).

11. Gene Transfer Promotes Bone Repair In Vivo

The present inventors surprisingly found that gene transfer into bone progenitor cells *in vivo* (i.e., cells in the regenerating tissue in the osteotomy gap) could be readily achieved. Currently, the preferred methods for achieving gene transfer generally involve using a fibrous

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collagen implant material soaked in a solution of DNA shortly before being placed in the site in which one desires to promote bone growth. As the studies presented herein show, the implant material facilitates the uptake of exogenous plasmid constructs by cells (in the osteotomy gap) which clearly participate in bone regeneration/repair. The transgenes, following cellular uptake, direct the expression of recombinant polypeptides, as evidenced by the *in vivo* expression of functional marker gene products.

Further studies are presented herein demonstrating that the transfer of an osteotropic gene results in cellular expression of a recombinant osteotropic molecule, which expression is directly associated with stimulation of new bone formation. After considering a relatively large number of candidate genes, a gene transfer vector coding for a fragment of human parathyroid hormone (hPTH1-34) was chosen for the inventors' initial studies. Several factors were considered in making this choice: (a), recombinant hPTH1-34 peptides can be discriminated from any endogenous rat hormone present in osteotomy tissues; (b), hPTH1-34 peptides will stimulate new bone formation in Sprague-Dawley rats, indicating that the human peptide can efficiently bind the PTH/PTHrP receptor on the rat osteoblast cell surface; and (c), there is only one PTH/PTHrP receptor, the gene for this receptor has been cloned, and cDNA probes to the receptor are available.

Thus, in terms of understanding the mechanism of action of the transgene on new bone formation *in vivo*, the inventors reasoned it most straightforward to correlate the expression of recombinant hPTH1-34 peptide and its receptor with new bone formation in the rat osteotomy model. Of course, following these initial studies, it is contemplated that any one of a wide

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variety of genes may be employed in connection with the bone gene transfer embodiments of the present invention.

Previous studies have indicated that hPTH1-34 is a
5 more powerful anabolic agent when given intermittently as
opposed to continuously. Despite the fact that an
anabolic effect would still be expected with continuous
dosing, as documented by the studies of Parsons and co-
workers (Tam et al., 1982; Spencer et al., 1989), there
10 was a concern that the PLJ-hPTH1-34 transgene may not
function very effectively as transfected cells would be
expected to express recombinant hPTH1-34 molecules in a
constitutive manner. The finding that transfection and
expression of the LPH-hPTH1-34 transgene did effectively
15 stimulate bone formation in the rat osteotomy model was
therefore an important result.

As the osteotomy site in this model is highly
vascularized, one possible complication of the studies
20 with the PLJ-hPTH1-34 transgene is the secretion of
recombinant human PTH from the osteotomy site with
consequent hypercalcemia and (potentially) animal death.
Weekly serum calcium levels should therefore be
determined when using this transgene. The fact that no
25 evidence of disturbed serum calcium levels has been found
in this work is therefore a further encouraging finding.

These studies complement others by the inventors in
which direct gene transfer was employed to introduce
30 genes into Achilles' tendon and cruciate ligament, as
described in Example XI.

Various immediate applications for using nucleic
acid delivery in connection with bone disorders became
35 apparent to the inventors following these surprising
findings. The direct transfer of an osteotropic gene to
promote fracture repair in clinical orthopaedic practice

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is just one use. Other important aspects of this technology include the use of gene transfer to treat patients with "weak bones", such as in diseases like osteoporosis; to improve poor healing which may arise for unknown reasons, e.g., fibrous non-union; to promote implant integration and the function of artificial joints; to stimulate healing of other skeletal tissues such as Achilles' tendon; and as an adjuvant to repair large defects. In all such embodiments, DNA is being used as a direct pharmaceutical agent.

12. Biological Functional Equivalents

As mentioned above, modification and changes may be made in the structure of an osteotropic gene and still obtain a functional molecule that encodes a protein or polypeptide with desirable characteristics. The following is a discussion based upon changing the amino acids of a protein to create an equivalent, or even an improved, second-generation molecule. The amino acid changes may be achieved by changing the codons of the DNA sequence, according to the following codon table:

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Table 1

Amino Acids		Codons					
	Alanine	Ala	A	GCA	GCC	GCG	GCU
	Cysteine	Cys	C	UGC	UGU		
5	Aspartic acid	Asp	D	GAC	GAU		
	Glutamic acid	Glu	E	GAA	GAG		
	Phenylalanine	Phe	F	UUC	UUU		
	Glycine	Gly	G	GGA	GGC	GGG	GGU
	Histidine	His	H	CAC	CAU		
10	Isoleucine	Ile	I	AUA	AUC	AUU	
	Lysine	Lys	K	AAA	AAG		
	Leucine	Leu	L	UUA	UUG	CUA	CUC CUG CUU
	Methionine	Met	M	AUG			
	Asparagine	Asn	N	AAC	AAU		
15	Proline	Pro	P	CCA	CCC	CCG	CCU
	Glutamine	Gln	Q	CAA	CAG		
	Arginine	Arg	R	AGA	AGG	CGA	CGC CGG CGU
	Serine	Ser	S	AGC	AGU	UCA	UCC UCG UCU
	Threonine	Thr	T	ACA	ACC	ACG	ACU
20	Valine	Val	V	GUA	GUC	GUG	GUU
	Tryptophan	Trp	W	UGG			
	Tyrosine	Tyr	Y	UAC	UAU		

25 For example, certain amino acids may be substituted
for other amino acids in a protein structure without
appreciable loss of interactive binding capacity with
structures such as, for example, antigen-binding regions
of antibodies or binding sites on substrate molecules.
30 Since it is the interactive capacity and nature of a
protein that defines that protein's biological functional
activity, certain amino acid sequence substitutions can
be made in a protein sequence, and, of course, its
underlying DNA coding sequence, and nevertheless obtain a
35 protein with like properties. It is thus contemplated by
the inventors that various changes may be made in the DNA
sequences of osteotropic genes without appreciable loss
of their biological utility or activity.

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In making such changes, the hydropathic index of amino acids may be considered. The importance of the hydropathic amino acid index in conferring interactive biologic function on a protein is generally understood in the art (Kyte and Doolittle, 1982, incorporate herein by reference). It is accepted that the relative hydropathic character of the amino acid contributes to the secondary structure of the resultant protein, which in turn defines the interaction of the protein with other molecules, for example, enzymes, substrates, receptors, DNA, antibodies, antigens, and the like.

Each amino acid has been assigned a hydropathic index on the basis of their hydrophobicity and charge characteristics (Kyte and Doolittle, 1982), these are: Isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

It is known in the art that certain amino acids may be substituted by other amino acids having a similar hydropathic index or score and still result in a protein with similar biological activity, i.e., still obtain a biological functionally equivalent protein. In making such changes, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity. U.S. Patent 4,554,101, incorporated herein by reference, states that the

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greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein.

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As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 \pm 1); glutamate (+3.0 \pm 1); serine (+0.3);
10 asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 \pm 1); alanine (-0.5); histidine *-0.5; cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

15

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent, and in particular, an immunologically equivalent protein. In
20 such changes, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those which are within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

25

As outlined above, amino acid substitutions are generally therefore based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. Exemplary substitutions which take various of
30 the foregoing characteristics into consideration are well known to those of skill in the art and include: arginine and lysine; glutamate and aspartate; serine and threonine; glutamine and asparagine; and valine, leucine and isoleucine.

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13. Site-Specific Mutagenesis

Site-specific mutagenesis is a technique useful in the preparation of individual peptides, or biologically functional equivalent proteins or peptides, through specific mutagenesis of the underlying DNA. The technique further provides a ready ability to prepare and test sequence variants, for example, incorporating one or more of the foregoing considerations, by introducing one or more nucleotide sequence changes into the DNA. Site-specific mutagenesis allows the production of mutants through the use of specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent nucleotides, to provide a primer sequence of sufficient size and sequence complexity to form a stable duplex on both sides of the deletion junction being traversed. Typically, a primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction of the sequence being altered.

In general, the technique of site-specific mutagenesis is well known in the art, as exemplified by various publications. As will be appreciated, the technique typically employs a phage vector which exists in both a single stranded and double stranded form. Typical vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage are readily commercially available and their use is generally well known to those skilled in the art. Double stranded plasmids are also routinely employed in site directed mutagenesis which eliminates the step of transferring the gene of interest from a plasmid to a phage.

In general, site-directed mutagenesis in accordance herewith is performed by first obtaining a single-stranded vector or melting apart of two strands of a

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double stranded vector which includes within its sequence a DNA sequence which encodes the desired osteotropic protein. An oligonucleotide primer bearing the desired mutated sequence is prepared, generally synthetically.

5 This primer is then annealed with the single-stranded vector, and subjected to DNA polymerizing enzymes such as *E. coli* polymerase I Klenow fragment, in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed wherein one strand encodes
10 the original non-mutated sequence and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate cells, such as *E. coli* cells, and clones are selected which include recombinant vectors bearing the mutated sequence arrangement.

15 The preparation of sequence variants of the selected osteotropic gene using site-directed mutagenesis is provided as a means of producing potentially useful species and is not meant to be limiting as there are
20 other ways in which sequence variants of osteotropic genes may be obtained. For example, recombinant vectors encoding the desired osteotropic gene may be treated with mutagenic agents, such as hydroxylamine, to obtain sequence variants.

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14. Monoclonal Antibody Generation

Means for preparing and characterizing antibodies are well known in the art (See, e.g., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, 1988; incorporated herein by reference).

The methods for generating monoclonal antibodies (MAbs) generally begin along the same lines as those for preparing polyclonal antibodies. Briefly, a polyclonal antibody is prepared by immunizing an animal with an immunogenic composition in accordance with the present invention and collecting antisera from that immunized animal. A wide range of animal species can be used for the production of antisera. Typically the animal used for production of anti-antisera is a rabbit, a mouse, a rat, a hamster, a guinea pig or a goat. Because of the relatively large blood volume of rabbits, a rabbit is a preferred choice for production of polyclonal antibodies.

As is well known in the art, a given composition may vary in its immunogenicity. It is often necessary therefore to boost the host immune system, as may be achieved by coupling a peptide or polypeptide immunogen to a carrier. Exemplary and preferred carriers are keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA). Other albumins such as ovalbumin, mouse serum albumin or rabbit serum albumin can also be used as carriers. Means for conjugating a polypeptide to a carrier protein are well known in the art and include glutaraldehyde, m-maleimidobencoyl-N-hydroxysuccinimide ester, carbodiimide and bis-biazotized benzidine.

As is also well known in the art, the immunogenicity of a particular immunogen composition can be enhanced by the use of non-specific stimulators of the immune response, known as adjuvants. Exemplary and preferred

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adjuvants include complete Freund's adjuvant (a non-specific stimulator of the immune response containing killed *Mycobacterium tuberculosis*), incomplete Freund's adjuvants and aluminum hydroxide adjuvant.

5

The amount of immunogen composition used in the production of polyclonal antibodies varies upon the nature of the immunogen as well as the animal used for immunization. A variety of routes can be used to
10 administer the immunogen (subcutaneous, intramuscular, intradermal, intravenous and intraperitoneal). The production of polyclonal antibodies may be monitored by sampling blood of the immunized animal at various points following immunization. A second, booster injection, may
15 also be given. The process of boosting and titering is repeated until a suitable titer is achieved. When a desired level of immunogenicity is obtained, the immunized animal can be bled and the serum isolated and stored, and/or the animal can be used to generate MAbs.

20

MAbs may be readily prepared through use of well-known techniques, such as those exemplified in U.S. Patent 4,196,265, incorporated herein by reference. Typically, this technique involves immunizing a suitable
25 animal with a selected immunogen composition, e.g., a purified or partially purified LTBP-3 protein, polypeptide or peptide. The immunizing composition is administered in a manner effective to stimulate antibody producing cells. Rodents such as mice and rats are
30 preferred animals, however, the use of rabbit, sheep frog cells is also possible. The use of rats may provide certain advantages (Goding, 1986, pp. 60-61), but mice are preferred, with the BALB/c mouse being most preferred

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as this is most routinely used and generally gives a higher percentage of stable fusions.

Following immunization, somatic cells with the
5 potential for producing antibodies, specifically B
lymphocytes (B cells), are selected for use in the MAb
generating protocol. These cells may be obtained from
biopsied spleens, tonsils or lymph nodes, or from a
peripheral blood sample. Spleen cells and peripheral
10 blood cells are preferred, the former because they are a
rich source of antibody-producing cells that are in the
dividing plasmablast stage, and the latter because
peripheral blood is easily accessible. Often, a panel of
animals will have been immunized and the spleen of animal
15 with the highest antibody titer will be removed and the
spleen lymphocytes obtained by homogenizing the spleen
with a syringe. Typically, a spleen from an immunized
mouse contains approximately 5×10^7 to 2×10^8
lymphocytes.

20
The antibody-producing B lymphocytes from the
immunized animal are then fused with cells of an immortal
myeloma cell, generally one of the same species as the
animal that was immunized. Myeloma cell lines suited for
25 use in hybridoma-producing fusion procedures preferably
are non-antibody-producing, have high fusion efficiency,
and enzyme deficiencies that render them incapable of
growing in certain selective media which support the
growth of only the desired fused cells (hybridomas).

30
Any one of a number of myeloma cells may be used, as
are known to those of skill in the art (Goding, pp.
65-66, 1986; Campbell, pp. 75-83, 1984). For example,
where the immunized animal is a mouse, one may use
35 P3-X63/Ag8, X63-Ag8.653, NS1/1.Ag 4 1, Sp210-Ag14, FO,
NSO/U, MPC-11, MPC11-X45-GTG 1.7 and S194/5XX0 Bul; for
rats, one may use R210.RCY3, Y3-Ag 1.2.3, IR983F and

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4B210; and U-266, GM1500-GRG2, LICR-LON-HMy2 and UC729-6 are all useful in connection with human cell fusions.

One preferred murine myeloma cell is the NS-1 myeloma cell line (also termed P3-NS-1-Ag4-1), which is readily available from the NIGMS Human Genetic Mutant Cell Repository by requesting cell line repository number GM3573. Another mouse myeloma cell line that may be used is the 8-azaguanine-resistant mouse murine myeloma SP2/0 non-producer cell line.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually comprise mixing somatic cells with myeloma cells in a 2:1 proportion, though the proportion may vary from about 20:1 to about 1:1, respectively, in the presence of an agent or agents (chemical or electrical) that promote the fusion of cell membranes. Fusion methods using Sendai virus have been described by Kohler and Milstein (1975; 1976), and those using polyethylene glycol (PEG), such as 37% (v/v) PEG, by Gefter et al. (1977). The use of electrically induced fusion methods is also appropriate (Goding pp. 71-74, 1986).

Fusion procedures usually produce viable hybrids at low frequencies, about 1×10^{-6} to 1×10^{-8} . However, this does not pose a problem, as the viable, fused hybrids are differentiated from the parental, unfused cells (particularly the unfused myeloma cells that would normally continue to divide indefinitely) by culturing in a selective medium. The selective medium is generally one that contains an agent that blocks the *de novo* synthesis of nucleotides in the tissue culture media. Exemplary and preferred agents are aminopterin, methotrexate, and azaserine. Aminopterin and methotrexate block *de novo* synthesis of both purines and pyrimidines, whereas azaserine blocks only purine

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synthesis. Where aminopterin or methotrexate is used, the media is supplemented with hypoxanthine and thymidine as a source of nucleotides (HAT medium). Where azaserine is used, the media is supplemented with hypoxanthine.

5

The preferred selection medium is HAT. Only cells capable of operating nucleotide salvage pathways are able to survive in HAT medium. The myeloma cells are defective in key enzymes of the salvage pathway, e.g., hypoxanthine phosphoribosyl transferase (HPRT), and they cannot survive. The B cells can operate this pathway, but they have a limited life span in culture and generally die within about two weeks. Therefore, the only cells that can survive in the selective media are those hybrids formed from myeloma and B cells.

10

15

This culturing provides a population of hybridomas from which specific hybridomas are selected. Typically, selection of hybridomas is performed by culturing the cells by single-clone dilution in microtiter plates, followed by testing the individual clonal supernatants (after about two to three weeks) for the desired reactivity. The assay should be sensitive, simple and rapid, such as radioimmunoassays, enzyme immunoassays, cytotoxicity assays, plaque assays, dot immunobinding assays, and the like.

20

25

The selected hybridomas would then be serially diluted and cloned into individual antibody-producing cell lines, which clones can then be propagated indefinitely to provide MABs. The cell lines may be exploited for MAB production in two basic ways. A sample of the hybridoma can be injected (often into the peritoneal cavity) into a histocompatible animal of the type that was used to provide the somatic and myeloma cells for the original fusion. The injected animal develops tumors secreting the specific monoclonal

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antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can then be tapped to provide MAbs in high concentration. The individual cell lines could also be cultured in vitro, where the MAbs are naturally secreted into the culture medium from which they can be readily obtained in high concentrations. MAbs produced by either means may be further purified, if desired, using filtration, centrifugation and various chromatographic methods such as HPLC or affinity chromatography.

15. LTBP-3

Other aspects of the present invention concern isolated DNA segments and recombinant vectors encoding LTBP-3, and the creation and use of recombinant host cells through the application of DNA technology, that express LTBP-3 gene products. As such, the invention concerns DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:3. These DNA segments are represented by those that include a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2 (FIG. 25). Compositions that include a purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3 (FIG. 26) are also encompassed by the invention.

30

The TGF- β s represent a family of structurally related molecules with diverse effects on mammalian cell shape, growth, and differentiation (Roberts and Sporn, 1990). Initially synthesized as a precursor consisting of an amino-terminal propeptide followed by mature TGF- β , two chains of nascent pro-TGF- β associate in most tissues to form a Mr ~106,000 inactive disulfide-bonded dimer.

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Homodimers are most common, but heterodimers have also been described (Cheifetz et al., 1987; Ogawa et al., 1992). During biosynthesis the mature TGF- β dimer is cleaved from the propeptide dimer. TGF- β latency results in part from the non-covalent association of propeptide and mature TGF- β dimers (Pircher et al., 1984, 1986; Wakefield et al., 1987; Millan et al., 1992; Miyazono and Heldin, 1989). Consequently, the propeptide dimer is often referred to as the latency associated protein (LAP), and LAP plus the disulfide-bonded TGF- β dimer are also known as the small latent complex. In the extracellular space small latent complexes must be dissociated to activate mature TGF- β . The mechanism of activation of the latent complex is thought to be one of the most important steps governing TGF- β effects (Lyons et al., 1988; Antonelli-Orlidge et al., 1989; Twardzik et al., 1990; Sato et al., 1993).

In certain lines of cultured cells small latent growth factor complexes may contain additional high molecular weight proteins. The best characterized of these high molecular weight proteins is the latent TGF- β binding protein, or LTBP (Miyazono et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990; Olofsson et al., 1992; Taketazu et al., 1994). LTBP produced by different cell types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990). Latent TGF- β complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF- β , but rather it is linked by a disulfide bond to LAP.

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Regarding the novel protein LTBP-3, the present invention concerns DNA segments, that can be isolated from virtually any mammalian source, that are free from total genomic DNA and that encode proteins having LTBP-3-like activity. DNA segments encoding LTBP-3-like species may prove to encode proteins, polypeptides, subunits, functional domains, and the like.

As used herein, the term "DNA segment" refers to a DNA molecule that has been isolated free of total genomic DNA of a particular species. Therefore, a DNA segment encoding LTBP-3 refers to a DNA segment that contains LTBP-3 coding sequences yet is isolated away from, or purified free from, total genomic DNA of the species from which the DNA segment is obtained. Included within the term "DNA segment", are DNA segments and smaller fragments of such segments, and also recombinant vectors, including, for example, plasmids, cosmids, phagemids, phage, viruses, and the like.

Similarly, a DNA segment comprising an isolated or purified LTBP-3 gene refers to a DNA segment including LTBP-3 coding sequences and, in certain aspects, regulatory sequences, isolated substantially away from other naturally occurring genes or protein encoding sequences. In this respect, the term "gene" is used for simplicity to refer to a functional protein, polypeptide or peptide encoding unit. As will be understood by those in the art, this functional term includes both genomic sequences, cDNA sequences and smaller engineered gene segments that express, or may be adapted to express, proteins, polypeptides or peptides.

"Isolated substantially away from other coding sequences" means that the gene of interest, in this case, a gene encoding LTBP-3, forms the significant part of the

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coding region of the DNA segment, and that the DNA segment does not contain large portions of naturally-occurring coding DNA, such as large chromosomal fragments or other functional genes or cDNA coding regions. Of course, this refers to the DNA segment as originally isolated, and does not exclude genes or coding regions later added to the segment by the hand of man.

In particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that encode an LTBP-3 species that includes within its amino acid sequence an amino acid sequence essentially as set forth in SEQ ID NO:3. In other particular embodiments, the invention concerns isolated DNA segments and recombinant vectors incorporating DNA sequences that include within their sequence a nucleotide sequence essentially as set forth in SEQ ID NO:2.

The term "a sequence essentially as set forth in SEQ ID NO:3" means that the sequence substantially corresponds to a portion of SEQ ID NO:3 and has relatively few amino acids that are not identical to, or a biologically functional equivalent of, the amino acids of SEQ ID NO:3. The term "biologically functional equivalent" is well understood in the art and is further defined in detail herein (for example, see section 7, preferred embodiments). Accordingly, sequences that have between about 70% and about 80%; or more preferably, between about 81% and about 90%; or even more preferably, between about 91% and about 99%; of amino acids that are identical or functionally equivalent to the amino acids of SEQ ID NO:3 will be sequences that are "essentially as set forth in SEQ ID NO:3".

In certain other embodiments, the invention concerns isolated DNA segments and recombinant vectors that

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include within their sequence a nucleic acid sequence essentially as set forth in SEQ ID NO:2. The term "essentially as set forth in SEQ ID NO:2" is used in the same sense as described above and means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:2 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:2. Again, DNA segments that encode proteins exhibiting LTBP-3-like activity will be most preferred.

It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N- or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region or may include various internal sequences, i.e., introns, which are known to occur within genes.

Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:2. Nucleic acid sequences that are "complementary" are those that are capable of base-pairing according to the standard Watson-Crick complementarity rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:2,

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under relatively stringent conditions such as those described herein.

The nucleic acid segments of the present invention, regardless of the length of the coding sequence itself, may be combined with other DNA sequences, such as promoters, polyadenylation signals, additional restriction enzyme sites, multiple cloning sites, other coding segments, and the like, such that their overall length may vary considerably. It is therefore contemplated that a nucleic acid fragment of almost any length may be employed, with the total length preferably being limited by the ease of preparation and use in the intended recombinant DNA protocol. For example, nucleic acid fragments may be prepared that include a short contiguous stretch identical to or complementary to SEQ ID NO:2, such as about 14 nucleotides, and that are up to about 10,000 or about 5,000 base pairs in length, with segments of about 3,000 being preferred in certain cases. DNA segments with total lengths of about 1,000, about 500, about 200, about 100 and about 50 base pairs in length (including all intermediate lengths) are also contemplated to be useful.

It will be readily understood that "intermediate lengths", in these contexts, means any length between the quoted ranges, such as 14, 15, 16, 17, 18, 19, 20, etc.; 21, 22, 23, etc.; 30, 31, 32, etc.; 50, 51, 52, 53, etc.; 100, 101, 102, 103, etc.; 150, 151, 152, 153, etc.; including all integers through the 200-500; 500-1,000; 1,000-2,000; 2,000-3,000; 3,000-5,000; 5,000-10,000 ranges, up to and including sequences of about 12,001, 12,002, 13,001, 13,002 and the like.

It will also be understood that this invention is not limited to the particular nucleic acid and amino acid sequences of SEQ ID NO:2 and SEQ ID NO:3. Recombinant

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vectors and isolated DNA segments may therefore variously include the LTBP-3 coding regions themselves, coding regions bearing selected alterations or modifications in the basic coding region, or they may encode larger polypeptides that nevertheless include LTBP-3-coding regions or may encode biologically functional equivalent proteins or peptides that have variant amino acids sequences.

10 The DNA segments of the present invention encompass biologically functional equivalent LTBP-3 proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the antigenicity of the protein or to test mutants in order to examine activity at the molecular level.

30 If desired, one may also prepare fusion proteins and peptides, e.g., where the LTBP-3 coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (e.g., proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively).

35 Recombinant vectors form further aspects of the present invention. Particularly useful vectors are contemplated to be those vectors in which the coding

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portion of the DNA segment, whether encoding a full length protein or smaller peptide, is positioned under the control of a promoter. The promoter may be in the form of the promoter that is naturally associated with a LTBP-3 gene, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment or exon, for example, using recombinant cloning and/or PCR™ technology, in connection with the compositions disclosed herein.

10

In other embodiments, it is contemplated that certain advantages will be gained by positioning the coding DNA segment under the control of a recombinant, or heterologous, promoter. As used herein, a recombinant or heterologous promoter is intended to refer to a promoter that is not normally associated with an LTBP-3 gene in its natural environment. Such promoters may include LTBP-3 promoters normally associated with other genes, and/or promoters isolated from any bacterial, viral, eukaryotic, or mammalian cell. Naturally, it will be important to employ a promoter that effectively directs the expression of the DNA segment in the cell type, organism, or even animal, chosen for expression. The use of promoter and cell type combinations for protein expression is generally known to those of skill in the art of molecular biology, for example, see Sambrook et al., 1989. The promoters employed may be constitutive, or inducible, and can be used under the appropriate conditions to direct high level expression of the introduced DNA segment, such as is advantageous in the large-scale production of recombinant proteins or peptides. Appropriate promoter systems contemplated for use in high-level expression include, but are not limited

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to, the *Pichia* expression vector system (Pharmacia LKB Biotechnology) (see Example XVI herein).

5 In connection with expression embodiments to prepare recombinant LTBP-3 proteins and peptides, it is contemplated that longer DNA segments will most often be used, with DNA segments encoding the entire LTBP-3 protein or functional domains, subunits, etc. being most preferred. However, it will be appreciated that the use
10 of shorter DNA segments to direct the expression of LTBP-3 peptides or epitopic core regions, such as may be used to generate anti-LTBP-3 antibodies, also falls within the scope of the invention. DNA segments that encode peptide antigens from about 15 to about 50 amino acids in length,
15 or more preferably, from about 15 to about 30 amino acids in length are contemplated to be particularly useful.

The LTBP-3 gene and DNA segments may also be used in connection with somatic expression in an animal or in the
20 creation of a transgenic animal. Again, in such embodiments, the use of a recombinant vector that directs the expression of the full length or active LTBP-3 protein is particularly contemplated.

25 In addition to their use in directing the expression of the LTBP-3 protein, the nucleic acid sequences disclosed herein also have a variety of other uses. For example, they also have utility as probes or primers in nucleic acid hybridization embodiments. As such, it is
30 contemplated that nucleic acid segments that comprise a sequence region that consists of at least a 14 nucleotide long contiguous sequence that has the same sequence as, or is complementary to, a 14 nucleotide long contiguous sequence of SEQ ID NO:2 will find particular utility.
35 Longer contiguous identical or complementary sequences, e.g., those of about 20, 30, 40, 50, 100, 200, 500, 1000

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(including all intermediate lengths) and even up to full length sequences will also be of use in certain embodiments.

5 The ability of such nucleic acid probes to specifically hybridize to LTBP-3-encoding sequences will enable them to be of use in detecting the presence of complementary sequences in a given sample. However, other uses are envisioned, including the use of the
10 sequence information for the preparation of mutant species primers, or primers for use in preparing other genetic constructions.

15 Nucleic acid molecules having sequence regions consisting of contiguous nucleotide stretches of 10-14, 15-20, 30, 50, or even of 100-200 nucleotides or so, identical or complementary to SEQ ID NO:2, are particularly contemplated as hybridization probes for use in, e.g., Southern and Northern blotting. This would
20 allow LTBP-3 structural or regulatory genes to be analyzed, both in diverse cell types and also in various mammalian cells. The total size of fragment, as well as the size of the complementary stretch(es), will ultimately depend on the intended use or application of
25 the particular nucleic acid segment. Smaller fragments will generally find use in hybridization embodiments, wherein the length of the contiguous complementary region may be varied, such as between about 10-14 and about 100 nucleotides, but larger contiguous complementarity
30 stretches may be used, according to the length complementary sequences one wishes to detect.

35 The use of a hybridization probe of about 10-14 nucleotides in length allows the formation of a duplex molecule that is both stable and selective. Molecules having contiguous complementary sequences over stretches greater than 10 bases in length are generally preferred,

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though, in order to increase stability and selectivity of the hybrid, and thereby improve the quality and degree of specific hybrid molecules obtained. One will generally prefer to design nucleic acid molecules having gene-
5 complementary stretches of 15 to 20 contiguous nucleotides, or even longer where desired.

Hybridization probes may be selected from any portion of any of the sequences disclosed herein. All
10 that is required is to review the sequence set forth in SEQ ID NO:2 and to select any continuous portion of the sequence, from about 10-14 nucleotides in length up to and including the full length sequence, that one wishes to utilize as a probe or primer. The choice of probe and
15 primer sequences may be governed by various factors, such as, by way of example only, one may wish to employ primers from towards the termini of the total sequence.

The process of selecting and preparing a nucleic acid segment that includes a contiguous sequence from within SEQ ID NO:2 may alternatively be described as preparing a nucleic acid fragment. Of course, fragments may also be obtained by other techniques such as, e.g., by mechanical shearing or by restriction enzyme
25 digestion. Small nucleic acid segments or fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, as is commonly practiced using an automated oligonucleotide synthesizer. Also, fragments may be obtained by
30 application of nucleic acid reproduction technology, such as the PCR™ technology of U.S. Patent 4,603,102 (incorporated herein by reference), by introducing selected sequences into recombinant vectors for recombinant production, and by other recombinant DNA
35 techniques generally known to those of skill in the art of molecular biology.

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Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of LTBP-3 gene or cDNA fragments. Depending on the application envisioned, one will desire to employ varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of 50°C to 70°C. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand, and would be particularly suitable for isolating LTBP-3 genes.

Of course, for some applications, for example, where one desires to prepare mutants employing a mutant primer strand hybridized to an underlying template or where one seeks to isolate LTBP-3-encoding sequences from related species, functional equivalents, or the like, less stringent hybridization conditions will typically be needed in order to allow formation of the heteroduplex. In these circumstances, one may desire to employ conditions such as about 0.15 M to about 0.9 M salt, at temperatures ranging from 20°C to 55°C. Cross-hybridizing species can thereby be readily identified as positively hybridizing signals with respect to control hybridizations. In any case, it is generally appreciated that conditions can be rendered more stringent by the addition of increasing amounts of formamide, which serves to destabilize the hybrid duplex in the same manner as increased temperature. Thus, hybridization conditions can be readily manipulated, and thus will generally be a method of choice depending on the desired results.

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In certain embodiments, it will be advantageous to employ nucleic acid sequences of the present invention in combination with an appropriate means, such as a label, for determining hybridization. A wide variety of appropriate indicator means are known in the art, including fluorescent, radioactive, enzymatic or other ligands, such as avidin/biotin, which are capable of giving a detectable signal. In preferred embodiments, one will likely desire to employ a fluorescent label or an enzyme tag, such as urease, alkaline phosphatase or peroxidase, instead of radioactive or other environmental undesirable reagents. In the case of enzyme tags, colorimetric indicator substrates are known that can be employed to provide a means visible to the human eye or spectrophotometrically, to identify specific hybridization with complementary nucleic acid-containing samples.

In general, it is envisioned that the hybridization probes described herein will be useful both as reagents in solution hybridization as well as in embodiments employing a solid phase. In embodiments involving a solid phase, the test DNA (or RNA) is adsorbed or otherwise affixed to a selected matrix or surface. This fixed, single-stranded nucleic acid is then subjected to specific hybridization with selected probes under desired conditions. The selected conditions will depend on the particular circumstances based on the particular criteria required (depending, for example, on the G+C content, type of target nucleic acid, source of nucleic acid, size of hybridization probe, etc.). Following washing of the hybridized surface so as to remove nonspecifically bound probe molecules, specific hybridization is detected, or even quantitated, by means of the label.

The following examples are included to demonstrate preferred embodiments of the invention. It should be

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appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus
5 can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result
10 without departing from the spirit and scope of the invention.

EXAMPLE I

ANIMAL MODEL FOR ASSESSING NEW BONE FORMATION

15

As various animal models were not suitable for studying the effects of nucleic acid transfer on bone formation, the inventors employed the following model system. The important features of the rat osteotomy
20 model are as described in the following protocol (which is generally completed in 25-35 minutes).

The osteotomy was performed on one femur per animal. Right to left differences have not been apparent, but
25 such differences are monitored in these studies, since the limb receiving the osteotomy is randomized.

After pre-operative preparation (i.e., shaving and Betadine® scrub), adult male Sprague Dawley rats (~500
30 gm, retired male breeders) were anesthetized using a 3% halothane 97% oxygen mixture (700 ml/min. flow rate). A lateral approach to the femur was made on one limb. Utilizing specially designed surgical guides, four 1.2-mm diameter pins were screwed into the diaphysis after pre-
35 drilling with a high speed precision bit. A surgical template ensured precise and parallel placement of the pins. The order of pin placement was always the same:

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outer proximal first and then outer distal, inner proximal and inner distal (with "outer" and "inner" referring to the distance from the hip joint). Pin placement in the center of the femur was ensured by fluoroscopic imaging during pin placement. The external fixator was secured on the pins and a 1 mm or 2 mm segmental defect was created in the central diaphysis through an incision using a Hall Micro 100 Oscillating saw (#5053-60 Hall surgical blades) under constant irrigation. Other than the size of the segmental defect, there is no difference between the 5 mm and 2 mm osteotomy protocols (FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C, FIG. 6D, FIG. 7A, FIG. 7B, FIG. 8A, FIG. 8B, FIG. 8C).

The contents of the osteotomy site were irrigated with sterile saline and the fibrous collagen implant material, previously soaked in a solution of plasmid DNA or other DNA construct, if appropriate, was placed in situ. The wound was then closed in layers. Since the fixator provided the necessary stability no limitations on animal ambulation existed, and other supports were not required. The surgical protocol has been successfully performed on 53 animals to date, including 35 controls (Table 2 and FIG. 24). None of these animals have died and no significant adverse effects have been observed, other than complications that might be associated with surgical fracture repair. Minor complications that were experienced include 1 animal that developed a post-operative osteomyelitis and 1 animal in which 2/4 pins loosened as a consequence of post-operative bone fracture.

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EXAMPLE II

IMPLANT MATERIAL FOR USE IN BONE GENE TRANSFER

Various implant materials may be used for
5 transferring genes into the site of bone repair and/or
regeneration *in vivo*. These materials are soaked in a
solution containing the DNA or gene that is to be
transferred to the bone regrowth site. Alternatively,
DNA may be incorporated into the matrix as a preferred
10 method of making.

One particular example of a suitable material is
fibrous collagen, which may be lyophilized following
extraction and partial purification from tissue and then
15 sterilized. A particularly preferred collagen is the
fibrous collagen implant material termed UltraFiber™, as
may be obtained from Norian Corp., (Mountain View, CA).
Detailed descriptions of the composition and use of
UltraFiber™ are provided in Gunasekaran et al., (1993a,
20 b; each incorporated herein by reference).

A more particularly preferred collagen is type II
collagen, with most particularly preferred collagen being
either recombinant type II collagen, or mineralized type
25 II collagen. Prior to placement in osteotomy sites,
implant materials are soaked in solutions of DNA (or
virus) under sterile conditions. The soaking may be for
any appropriate and convenient period, e.g., from 6
minutes to over-night. The DNA (e.g., plasmid) solution
30 will be a sterile aqueous solution, such as sterile water
or an acceptable buffer, with the concentration generally
being about 0.5 - 1.0 mg/ml. Currently preferred
plasmids are those such as pGL2 (Promega), pSV40 β -gal,
pAd.CMVlacZ, and pLJ.

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EXAMPLE III

PARATHYROID HORMONE GENE CONSTRUCTS

5 The active fragment of the human parathyroid hormone gene (hPTH1-34) was chosen as the first of the osteotropic genes to be incorporated into an expression vector for use in gene transfer to promote new bone formation in the rat osteotomy model.

10 The inventors chose to construct the hPTH1-34 transgene in the pLJ expression vector (FIG. 10), since this vector was appropriate for studies of transgene function both *in vitro* and *in vivo*. A schematic of the PLJ-hPTH1-34 transgene is shown in FIG. 10. The DNA and
15 amino acid sequences of the hPTH1-34 are well known, e.g., see Hendy et al., (1981, incorporated herein by reference). To insert the transgene into the PLJ expression vector PCR™ of a full-length PTH recombinant clone was employed, followed by standard molecular
20 biological manipulation.

A retroviral stock was then generated following CaPO₄-mediated transfection of ϕ crip cells with the PLJ-hPTH1-34 construct, all according to standard protocols
25 (Sambrook et al., 1989). Independent transduced Rat-1 clones were obtained by standard infection and selection procedures (Sambrook et al., 1989).

One clone (YZ-15) was analyzed by Southern analysis,
30 demonstrating that the PLJ-hPTH1-34 transgene had stably integrated into the Rat-1 genome (FIG. 11). A Northern analysis was next performed to show that the YZ-15 clone expressed the PLJ-hPTH1-34 transgene, as evidenced by the presence of specific PLJ-hPTH1-34 transcripts (FIG. 12).

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EXAMPLE IV

PARATHYROID HORMONE POLYPEPTIDE EXPRESSION AND ACTIVITY

A sensitive and specific radioimmunoassay was performed to demonstrate that the YZ-15 cells expressed and secreted a recombinant hPTH1-34 molecule (Table 2). The radioimmunoassay was performed on media from transduced Rat-1 clones. To quantify secretion of the recombinant hPTH-1-34 peptide produced by YZ-15 cells, the culture medium from one 100 mm confluent dish was collected over a 24 hour period and assayed with the NH₂-terminal hPTH RIA kit (Nichols Institute Diagnostics) according to the manufacturer's protocol. PLJ-hPTH1-87 cells and BAG cells served as positive and negative controls, respectively.

Protein concentrations in Table 2 are expressed as the average of three assays plus the standard deviation (in parenthesis). The concentration of the 1-34 and full length (1-84) peptides was determined relative to a standard curve generated with commercially available reagents (Nichols Institute Diagnostics).

Table 2

CELL LINES	PTH (pg/ml)
YZ-15	247 (± 38)
PLJ-hPTH1-84	2616 (± 372)
BAG	13 (± 3)

As shown in Table 2, PTH expression was detected in both YZ-15 cells and PLJ-hPTH1-84 cells. BAG cells produced no detectable PTH and served as a baseline for the RIA. These results demonstrate that YZ-15 cells expressed recombinant hPTH1-34 protein.

The recombinant hPTH1-34 molecule was added to rat

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osteosarcoma cells and a cAMP response assay conducted in order to determine whether the secreted molecule had biological activity. Unconcentrated media was collected from YZ-15 cells, PLJ-hPTH1-84 cells, and BAG cells and was used to treat ROS17/2.8 cells for 10 minutes, as described (Majmudar et al., 1991). cAMP was then extracted from treated cells and quantified by RIA (Table 3). The amount of cAMP shown is the average of three assays. The standard deviation of the mean is shown in parenthesis.

Table 3

CELL LINES	cAMP (pmol)
YZ-15	20.3 (\pm 0.25)
PLJ-hPTH184	88.5 (\pm 4.50)
BAG	7.6 (\pm 0.30)

A cAMP response was induced by the recombinant PTH secreted by the YZ-15 cells and by PLJ-hPTH1-84 cells. BAG cells produced no PTH and served as the baseline for the cAMP assay. These results provide direct in vitro evidence that the PLJ-hPTH1-34 transgene directs the expression and secretion of a functional osteotropic agent.

EXAMPLE V

BONE MORPHOGENETIC PROTEIN (BMP) GENE CONSTRUCTS

The murine bone morphogenetic protein-4 (BMP-4) was chosen as the next of the osteotropic genes to be incorporated into an expression vector for use in promoting bone repair and regeneration.

A full length murine BMP-4 cDNA was generated by screening a murine 3T3 cell cDNA library (Stratagene). The human sequence for BMP-4 is well known to those of

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skill in the art and has been deposited in Genbank. Degenerate oligonucleotide primers were prepared and employed in a standard PCR™ to obtain a murine cDNA sequence.

5

The ends of the cDNA clone were further modified using the polymerase chain reaction so that the full length cDNA (5'→3' direction) encodes the natural murine initiator Met codon, the full length murine coding sequence, a 9 amino acid tag (known as the HA epitope), and the natural murine stop codon. The amino acid sequence encoded by the murine BMP-4 transgene is shown in FIG. 24; this entire sequence, including the tag, is represented by SEQ ID NO:1.

10

15

Placement of the HA epitope at the extreme carboxy terminus should not interfere with the function of the recombinant molecule sequence *in vitro* or *in vivo*. The advantage of the epitope is for utilization in immunohistochemical methods to specifically identify the recombinant murine BMP-4 molecule in osteotomy tissues *in vivo*, e.g., the epitope can be identified using a commercially available monoclonal antibody (Boehringer-Mannheim), as described herein.

20

25

Studies to demonstrate that the murine BMP-4 transgene codes for a functional osteotropic agent include, for example, (a) transfection of COS cells and immunoprecipitation of a protein band of the correct size using a monoclonal anti-HA antibody (Boehringer-Mannheim); and (b) a quantitative *in vivo* bone induction bioassay (Sampath and Reddi, 1981) that involves implanting proteins from the medium of transfected COS cells beneath the skin of male rats and scoring for new bone formation in the ectopic site.

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EXAMPLE VI

DETECTION OF mRNA BY TISSUE IN SITU HYBRIDIZATION

The following technique describes the detection of mRNA in tissue obtained from the site of bone regeneration. This may be useful for detecting expression of the transgene mRNA itself, and also in detecting expression of hormone or growth factor receptors or other molecules. This method may be used in place of, or in addition to, Northern analyses, such as those described in FIG. 13.

DNA from a plasmid containing the gene for which mRNA is to be detected is linearized, extracted, and precipitated with ethanol. Sense and antisense transcripts are generated from 1 mg template with T3 and T7 polymerases, e.g., in the presence of [³⁵S] UTP at >6 mCi/ml (Amersham Corp., >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega), with the remaining *in vitro* transcription reagents provided in a kit (SureSite, Novagen Inc.). After transcription at 37°C for 1 hour, DNA templates are removed by a 15 minute digestion at 37°C with 0.5 U/ml RNase-free DNase I, extracted, and precipitated with ethanol. Riboprobes are hydrolyzed to an average final length of 150 bp by incubating in 40 mM NaHCO₃, 60 mM Na₂CO₃, 80 mM DTT at 60°C, according to previously determined formula. Hydrolysis is terminated by addition of sodium acetate, pH 6.0, and glacial acetic acid to 0.09 M and 0.005% (v/v), respectively, and the probes are then ethanol precipitated, dissolved in 0.1 M DTT, counted, and stored at -20°C until use.

RNase precautions are taken in all stages of slide preparation. Bouins fixed, paraffin embedded tissue sections are heated to 65°C for 10 minutes, deparaffinized in 3 changes of xylene for 5 minutes, and rehydrated in a descending ethanol series, ending in

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phosphate-buffered saline (PBS). Slides will be soaked in 0.2 N HCl for 5 min., rinsed in PBS, digested with 0.0002% proteinase K in PBS for 30 minutes at 37°C and rinsed briefly with DEPC-treated water. After

5 equilibrating for 3 minutes in 0.1 M triethanolamine-HCl (TEA-HCl), pH 8.0, sections are acetylated in 0.25% (v/v) acetic anhydride in 0.1 M TEA-HCl for 10 minutes at room temperature, rinsed in PBS, and dehydrated in an ascending ethanol series. Each section receives 100-200

10 ml prehybridization solution (0.5 mg/ml denatured RNase-free tRNA (Boehringer-Mannheim), 10 mM DTT, 5 mg/ml denatured, sulfurylated salmon sperm DNA, 50% formamide, 10% dextran sulfate, 300 mM NaCl, 1X RNase-free Denhardt's solution (made with RNase-free bovine serum

15 albumin, Sigma), 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) and then incubate on a 50°C slide warmer in a humidified enclosure for 2 hours. The sulfurylated salmon-sperm DNA blocking reagent is used in both prehybridization and hybridization solutions to help reduce nonspecific

20 binding to tissue by ³⁵S groups on the probe. It is prepared by labeling RNase-free salmon sperm DNA (Sigma) with non-radioactive α -thio-dCTP and α -thio-dATP (Amersham) in a standard random oligonucleotide-primed DNA labeling reaction. Excess prehybridization solution

25 is removed with a brief rinse in 4X SSC before application of probe.

Riboprobes, fresh tRNA and sulfurylated salmon sperm DNA will be denatured for 10 minutes at 70°C, and chilled

30 on ice. Hybridization solution, identical to prehybridization solution except with denatured probe added to 5×10^6 CPM/ml, is applied and slides incubated at 50°C overnight in sealed humidified chambers on a slide warmer. Sense and antisense probes are applied to

35 serial sections. Slides are rinsed 3 times in 4X SSC, washed with 2X SSC, 1 mM DTT for 30 min. at 50°C, digested with RNase A (20 mg/ml RNase A, 0.5 M NaCl, 10

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mM Tris, pH 8.0, 1 mM EDTA, pH 8.0) for 30 min. at 37°C, and rinsed briefly with 2X SSC, 1 mM DTT. Three additional washes are performed, each at 50°C for 30 minutes: once in 2X SSC, 50% formamide, 1 mM DTT, and
5 twice in 1X SSC, 0.13% (w/v) sodium pyrophosphate, 1 mM DTT.

Slides are dehydrated in an ascending ethanol series (with supplementation of the dilute ethanols (50% and
10 70%) with SSC and DTT to 0.1X and 1 mM, respectively). Slides are exposed to X-ray film for 20-60 hours to visualize overall hybridization patterns, dipped in autoradiographic emulsion (Kodak NTB-2, diluted to 50% with 0.3 M ammonium acetate), slowly dried for 2 hours,
15 and exposed (4°C) for periods ranging from 8 days to 8 weeks. After developing emulsion, sections are counter stained with hematoxylin and eosin, dehydrated, and mounted with xylene-based medium. The hybridization signal is visualized under darkfield microscopy.

20

The above *in situ* hybridization protocol may be used, for example, in detecting the temporal and spatial pattern of PTH/PTHrP receptor expression. A suitable rat PTH/PTHrP receptor cDNA probe (R15B) is one that consists
25 of a 1810 bp region encoding the full length rat bone PTH/PTHrP receptor (Abou-Samra et al., 1992). The cDNA fragment is subcloned into pcDNA 1 (Invitrogen Corp., San Diego, CA) and is cut out using *Xba*I and *Bam*HI. This probe has provided positive signals for northern blot
30 analysis of rat, murine, and human osteoblastic cell lines, rat primary calvarial cells, and murine bone tissue. The pcDNA I plasmid contains a T7 and SP6 promoter that facilitate the generation of cRNA probes for *in situ* hybridization. The full length transcript
35 has been used to detect PTH/PTHrP receptor in sections of bone (Lee et al., 1994). The PTHrP cDNA probe (Yasuda et al., 1989) is a 400 bp subcloned fragment in

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pBluescript (Stratagene). This probe has been used for *in situ* hybridization, generating an antisense cRNA probe using *Bam*HI cleavage and the T3 primer and a sense cRNA probe using *Eco*RI cleavage and the T7 primer.

5

EXAMPLE VII

IN VIVO PROTEIN DETECTION FOLLOWING TRANSGENE EXPRESSION

1. β -galactosidase Transgene

10

Bacterial β -galactosidase can be detected immunohistochemically. Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the bacterial β -galactosidase protein.

For immunohistochemistry, cross-Sections (2-3 mm thick) were transferred to poly-L-Lysine coated microscope slides and fixed in acetone at 0°C for at least 20 min. Sections were rehydrated in PBS. Endogenous peroxidase activity was quenched by immersion of tissue sections in 0.1% hydrogen peroxide (in 95% methanol) at room temperature for 10 min, and quenched sections were washed 3x in PBS. In some cases, sectioned calvariae were demineralized by immersion in 4% EDTA, 5% polyvinyl pyrrolidone, and 7% sucrose, pH 7.4, for 24 h at 4°C. Demineralized sections were washed 3x before application for antibodies. Primary antibodies were used without dilution in the form of hybridoma supernatant. Purified antibodies were applied to tissue sections at a concentration of 5 mg/ml. Primary antibodies were detected with biotinylated rabbit antimouse IgG and peroxidase conjugated streptavidin (Zymed Histostain-SPkit). After peroxidase staining, sections were counterstained with hematoxylin.

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Bacterial β -gal can also be detected by substrate utilization assays. This is conducted using commercially available kits (e.g., Promega) according to the manufacturers' instructions.

5

2. Luciferase Transgene

Luciferase can be detected by substrate utilization assays. This is conducted using commercially available kits (e.g., Promega) according to the manufacturers' instructions.

10

3. PTH Transgenes

15

Recombinant PTH, such as hPTH1-34 peptide, is assayed in homogenates of osteotomy gap tissue, for example, using two commercially available radioimmunoassay kits according to the manufacturer's protocols (Nichols Institute Diagnostics, San Juan Capistrano, CA).

20

One kit is the Intact PTH-Parathyroid Hormone 100T Kit. This radioimmunoassay utilizes an antibody to the carboxy terminus of the intact hormone, and thus is used to measure endogenous levels of hormone in gap osteotomy tissue. This assay may be used to establish a baseline value PTH expression in the rat osteotomy model.

25

The second kit is a two-site immunoradiometric kit for the measurement of rat PTH. This kit uses affinity purified antibodies specific for the amino terminus of the intact rat hormone (PTH1-34) and thus will measure endogenous PTH production as well as the recombinant protein. Previous studies have shown that these antibodies cross-react with human PTH and thus are able to recognize recombinant molecules in vivo.

30

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Values obtained with kit #1 (antibody to the carboxy terminus) are subtracted from values obtained with kit #2 (antibody to the amino terminus) to obtain an accurate and sensitive measurements. The level of recombinant peptide is thus correlated with the degree of new bone formation.

4. BMP Transgene

Preferably, BMP proteins, such as the murine BMP-4 transgene peptide product, are detected immunohistochemically using a specific antibody that recognizes the HA epitope (Majmudar et al., 1991), such as the monoclonal antibody available from Boehringer-Mannheim. Antibodies to BMP proteins themselves may also be used. Such antibodies, along with various immunoassay methods, are described in U.S. Patent 4,857,456, incorporated herein by reference.

Osteotomy tissue specimens are fixed in Bouins fixative, demineralized, and then split in half along the longitudinal plane. One-half of each specimen is embedded in paraffin for subsequent immunohistochemical identification of the recombinant murine BMP-4 molecule.

EXAMPLE VIII

DIRECT GENE TRANSFER INTO REGENERATING BONE IN VIVO

To assess the feasibility of direct gene transfer into regenerating bone *in vivo*, marker gene transfer into cells in the rat osteotomy model was employed. These studies involved two marker genes: bacterial β -galactosidase and insect luciferase.

Aliquots of a fibrous collagen implant material were soaked in solutions of pure marker gene DNA. The implant

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materials were then placed in the osteotomy site, and their expression determined as described above.

It was found that both marker genes were
5 successfully transferred and expressed, without any
failures, as demonstrated by substrate utilization assays
(FIG. 5A, FIG. 5B, FIG. 6A, FIG. 6B, FIG. 6C and FIG.
6D). Since mammalian cells do not normally synthesize
either marker gene product, this provides direct evidence
10 that osteotomy repair cells were transfected *in vivo* and
then expressed the β -galactosidase and luciferase
transgenes as a functional enzymes.

EXAMPLE IX

15 ADENOVIRAL GENE TRANSFER INTO REGENERATING BONE IN VIVO

One of the alternative methods to achieve *in vivo*
gene transfer into regenerating bone is to utilize an
adenovirus-mediated transfer event. Successful
20 adenoviral gene transfer of a marker gene construct into
bone repair cells in the rat osteotomy model has been
achieved (FIG. 23A, FIG. 23B, and FIG. 23C).

The inventors employed the adenoviral vector pAd.
25 CMVlacZ, which is an example of a replication-defective
adenoviral vector which can replicate in permissive cells
(Stratford-Perricaudet et al., 1992). In pAd.CMVlacZ,
the early enhancer/promoter of the cytomegalovirus (CMV)
is used to drive transcription of lacZ with an SV40
30 polyadenylation sequence cloned downstream from this
reporter (Davidson et al., 1993).

The vector pAd.RSV4 is also utilized by the
inventors. This vector essentially has the same backbone
35 as pAdCMVlacZ, however the CMV promoter and the single
BglII cloning site have been replaced in a cassette-like
fashion with BglII fragment that consists of an RSV

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promoter, a multiple cloning site, and a poly(A⁺) site. The greater flexibility of this vector is contemplated to be useful in subcloning osteotropic genes, such as the hPTH1-34 cDNA fragment, for use in further studies.

5

To generate recombinant PTH adenovirus, a 100-mm dish of 293 cells is transfected using calcium phosphate with 20 mg of a plasmid construct, e.g., the plasmid containing the hPTH1-34 insert linearized with NheI, plus 10 2 mg of wild type adenovirus DNA digested with XbaI and ClaI. The adenovirus DNA is derived from adenovirus type 5, which contains only a single XbaI and ClaI sites and has a partial deletion of the E3 region. Approximately 7 days post-transfection, cells and media are harvested and 15 a lysate prepared by repeated freeze-thaw cycles. This lysate is diluted and used to infect 60 -mm dishes of confluent 293 cells for 1 hour. The cells are then overlaid with 0.8% agar/1X MEM/2% calf serum/12.5 mM MgCl₂. Ten days post-infection, individual plaques are to 20 be picked and used to infect 60-mm dishes of 293 cells to expand the amount of virus. Positive plaques are selected for further purification and the generation of adenoviral stocks.

25 To purify recombinant adenovirus, 150-mm dishes of 75-90% confluent 293 cells are infected with 2-5 PFU/cell, a titer that avoids the potential cytotoxic effects of adenovirus. Thirty hours post-infection, the cells are rinsed, removed from the dishes, pelleted, and 30 resuspended in 10 mM Tris-HCl, pH 8.1. A viral lysate is generated by three freeze-thaw cycles, cell debris is removed by centrifugation for 10 min. at 2,000 rpm, and the adenovirus is purified by density gradient centrifugation. The adenovirus band is stored at -20°C 35 in sterile glycerol/BSA until needed.

The solution of virus particles was sterilized and

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incubated with the implant material (from 6 min to overnight), and the virus-impregnated material was implanted into the osteotomy gap; where viral infection of cells clearly occurred. The results obtained clearly demonstrated the exquisite specificity of the anti- β -gal antibody (Sambrook et al., 1989), and conclusively demonstrated expression of the marker gene product in chondrocyte-like cells of the osteotomy gap. The nuclear-targeted signal has also been observed in pre-osteoblasts.

EXAMPLE X

TRANSFER OF AN OSTEOTROPIC GENE STIMULATES BONE REGENERATION/REPAIR IN VIVO

In order for a parathyroid hormone (PTH) transgene to function as an osteotropic agent, it is likely that there is a requirement for the PTH/PTHrP receptor to be expressed in the bone repair tissue itself. Therefore, the inventors investigated PTH/PTHrP receptor expression in the rat osteotomy model.

A Northern analysis of poly-A(+) RNA was conducted which demonstrated that the PTH/PTHrP receptor was expression in osteotomy repair tissue (FIG. 13).

The inventors next investigated whether gene transfer could be employed to create transfected cells that constitutively express recombinant hPTH1-34 *in vivo*, and whether this transgene can stimulate bone formation. The rate of new bone formation is analyzed as follows. At necropsy the osteotomy site is carefully dissected for histomorphometric analysis. The A-P and M-L dimensions of the callus tissue are measured using calipers. Specimens are then immersion fixed in Bouins fixative, washed in ethanol, and demineralized in buffered formic acid. Plastic embedding of decalcified materials is used because of the superior dimensional stability of

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methacrylate during sample preparation and sectioning.

Tissue blocks are dehydrated in increasing alcohol concentrations and embedded. 5 mm thick sections are cut in the coronal plane using a Reichert Polycut microtome. Sections are prepared from midway through the width of the marrow cavity to guard against a sampling bias. Sections for light microscopy are stained using a modified Goldner's trichrome stain, to differentiate bone, osteoid, cartilage, and fibrous tissue. Sections are cover-slipped using Eukitt's mounting medium (Calibrated Instruments, Ardsley, NY). Histomorphometric analyses are performed under brightfield using a Nikon Optiphot Research microscope. Standard point count stereology techniques using a 10 mm x 10 mm eyepiece grid reticular are used.

Total callus area is measured at 125X magnification as an index of the overall intensity of the healing reaction. Area fractions of bone, cartilage, and fibrous tissue are measured at 250 X magnification to examine the relative contribution of each tissue to callus formation. Since the dimensions of the osteotomy gap reflect the baseline (time 0), a measurement of bone area at subsequent time intervals is used to indicate the rate of bone infill. Statistical significance is assessed using analysis of variance, with post-hoc comparisons between groups conducted using Tukey's studentized range t test.

In the 5-mm rat osteotomy model described above, it was found that PTH transgene expression can stimulate bone regeneration/repair in live animals (FIG. 6A, FIG. 6B, FIG. 6C, and FIG. 6D). This is a particularly important finding as it is known that hPTH1-34 is a more powerful anabolic agent when given intermittently as opposed to continuously, and it is the continuous-type delivery that results from the gene transfer methods used

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here.

Although the present inventors have already demonstrated success of direct gene transfer into regenerating bone *in vivo*, the use of *ex vivo* treatment protocols is also contemplated. In such embodiments, bone progenitor cells would be isolated from a particular animal or human subject and maintained in an *in vitro* environment. Suitable areas of the body from which to obtain bone progenitor cells are areas such as the bone tissue and fluid surrounding a fracture or other skeletal defect (whether or not this is an artificially created site) and from the bone marrow. Isolated cells would then be contacted with the DNA (or recombinant viral) composition, with, or preferably without, a matrix, when the cells would take up the DNA (or be infected by the recombinant virus). The stimulated cells would then be returned to the site in the animal or patient where bone repair is to be stimulated.

EXAMPLE XI

TRANSFER OF GENES TO ACHILLES' TENDON AND TO CRUCIATE LIGAMENT IN VIVO

The studies on regenerating bone described above complement others by the inventors in which gene transfer was successfully employed to introduce genes into Achilles' tendon (FIG 3A, FIG. 3B, FIG. 3C, FIG. 3D, and FIG. 3E) and cruciate ligament (FIG. 4).

The Achilles' tendon consist of cells and extracellular matrix organized in a characteristic tissue architecture. Tissue wounding can disrupt this architecture and stimulate a wound healing response. The wounded tendon will regenerate, as opposed to scar, if its connective tissue elements remain approximately intact. Regeneration is advantageous because scar tissue is not optimally designed to support normal mechanical

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function. Segmental defects in tendon due to traumatic injury may be treated with biological or synthetic implants that encourage neo-tendon formation. This strategy is limited, however, by the availability of effective (autologous) biological grafts, the long term stability and compatibility of synthetic prostheses, and the slow rate of incorporation often observed with both types of implants.

The inventors hypothesized that the effectiveness of biological grafts may be enhanced by the over-expression of molecules that regulate the tissue regeneration response. Toward this end, they developed a model system in which segmental defects in Achilles' tendon are created and a novel biomaterial, is used as a tendon implant/molecular delivery agent. In the present example, the ability to deliver and express marker gene constructs into regenerating tendon tissue is demonstrated.

Plasmid (pSV β gal, Promega) stock solutions were prepared according to standard protocols (Sambrook et al., 1989). SIS graft material was prepared from a segment of jejunum of adult pigs (Badylak et al., 1989). At harvest, mesenteric tissues were removed, the segment was inverted, and the mucosa and superficial submucosa were removed by a mechanical abrasion technique. After returning the segment to its original orientation, the serosa and muscle layers were rinsed, sterilized by treatment with dilute peracetic acid, and stored at 4°C until use.

Mongrel dogs (all studies) were anesthetized, intubated, placed in right-lateral recumbency upon a heating pad, and maintained with inhalant anesthesia. A lateral incision from the musculotendinous junction to the plantar fascia was used to expose the Achilles'

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tendon. A double thickness sheet of SIS was wrapped around a central portion of the tendon, both ends were sutured, a 1.5 cm segment of the tendon was removed through a lateral opening in the graft material, and the graft and surgical site were closed. The leg was immobilized for 6 weeks and then used freely for 6 weeks. Graft tissues were harvested at time points indicated below, fixed in Bouins solution, and embedded in paraffin. Tissue sections (8 μ m) were cut and used for immunohistochemistry.

In an initial study, SIS material alone (SIS-alone graft) engrafted and promoted the regeneration of Achilles' tendon following the creation of a segmental defect in mongrel dogs as long as 6 months post surgery. The remodeling process involved the rapid formation of granulation tissue and eventual degradation of the graft. Scar tissue did not form, and evidence of immune-mediated rejection was not observed.

In a second study, SIS was soaked in a plasmid DNA solution (SIS+plasmid graft) and subsequently implanted as an Achilles' tendon graft (n=2 dogs) or a cruciate ligament graft (n=2 dogs) in normal mongrel dogs. A pSV β gal plasmid that employs simian virus 40 regulatory sequences to drive β -galactosidase (β -gal) activity was detectable by immunohistochemistry using a specific antibody in 4/4 animals. As a negative control, β -gal activity was not detected in the unoperated Achilles' tendon and cruciate ligament of these animals. It appeared, therefore, that SIS facilitated the uptake and subsequent expression of plasmid DNA by wound healing cells in both tendon and ligament.

A third study was designed to evaluate the time course of β -gal transgene expression. SIS + plasmid grafts were implanted for 3, 6, 9, and 12 weeks (n=2 dogs

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pr time point) and transgene expression was assayed by immunohistochemistry and by *in situ* hybridization. Cross-sections (8- μ m) of Bouins fixed, paraffin embedded tissue were cut and mounted on ProbeOn Plus slides (Fisher). Immunohistochemistry was performed according to the protocol provided with the Histostain-SP kit (Zymed). In brief, slides were incubated with a well characterized anti- β -galactosidase antibody (1:200 dilution, 5'→3'), washed in PBS, incubated with a biotinylated second antibody, washed, stained with the enzyme conjugate plus a substrate-chromogen mixture, and then counterstained with hematoxylin and eosin.

Bacterial β -gal activity was detected in tendons that received the SIS+plasmid graft (8/8 animals). Although not rigorously quantitative, transgene expression appeared to peak at 9-12 weeks. Bacterial β -gal gene expression was not detected in animals that received SIS-alone grafts (N=2, 3 weeks and 12 weeks). Again, scar tissue did not form and evidence of immune-mediated rejection was not observed.

This study demonstrated that the mucosal biomaterial SIS can function as an autologous graft that promotes the regeneration of tissues such as Achilles' tendon and anterior cruciate ligament. SIS can also be used to deliver a marker gene construct to regenerating tissue.

EXAMPLE XIII

MECHANICAL PROPERTIES OF NEW BONE FORMATION

The mechanical properties of new bone formed during gene transfer may be measured using, e.g., whole bone torsion tests which create a stress state in which the maximum tensile stresses will occur on planes that lie obliquely to the bone's longitudinal axis. Such tests may provide important inferences about the mechanical

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anisotropy of callus tissue and the degree of osseous integration of new bone tissue. These tests are particularly advantageous in the evaluation of fracture specimens, e.g., the irregular shape of callus tissue typically precludes the use of whole bone 4-point bending tests because it is impossible to reproducibly align the points from specimen to specimen.

Femurs are tested on an MTS Servohydraulic Testing Machine while moist and at room temperature. A torque sensor and rotary variable displacement transducer provides data for torque-angular displacement curves. Specially designed fixtures support each bone near the metaphyseal-diaphyseal junctions, and apply a 2-point load to the diaphysis. Tests are conducted at a constant rate of displacement equal to 20 degrees/sec. A 250 inch-ounce load cell measures the total applied force. All bones are tested while moist and room temperature. Torque and angular displacement data are acquired using an analog-to-digital converter and a Macintosh computer and software. From this data, the following variables are calculated: a) maximum torque, b) torsional stiffness, the slope of the pre-yield portion of the curve determined from a linear regression of the data, c) energy to failure, the area under the torque-angular displacement curve to the point of failure, and d) the angular displacement ratio, the ratio of displacement at failure to displacement at yield. Statistical significance is determined Analysis of Variance followed by multiple comparisons with appropriate corrections (e.g., Bonferroni).

This invention also provides a means of using osteotropic gene transfer in connection with reconstructive surgery and various bone remodelling procedures. The techniques described herein may thus be employed in connection with the technology described by

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Yasko et al., 1992; Chen et al., 1991; and Beck et al., 1991, each incorporated herein by reference.

EXAMPLE XIV

5 TYPE II COLLAGEN PROMOTES NEW BONE GROWTH

10 Certain matrix materials are capable of stimulating at least some new growth in their own right, i.e., are "osteoconductive materials". Potential examples of such materials are well known in the field of orthopedic research and include preparations of hydroxyapatite; preparations of crushed bone and mineralized collagen; PLGA block copolymers and polyanhydride. The ability of these materials to stimulate new bone formation
15 distinguishes them from inert implant materials such as methylcellulose, which have in the past been used to deliver BMPs to sites of fracture repair.

20 This Example relates to a study using the rat osteotomy model with implants made of collagen type I (Sigma), collagen type II (Sigma), and UltraFiber™ (Norian Corp.). These materials have been placed in situ without DNA of any type. Five animals received an osteotomy with 10 mg of a type II collagen implant alone
25 (10 mg refers to the original quantity of lyophilized collagen). Five of five control animals received an osteotomy with 10 mg of a type I collagen implant alone. Animals were housed for three weeks after surgery and then sacrificed.

30 The results of these studies were that SIS appeared to retard new bone formation; type I collagen incited a moderately intense inflammatory response; and UltraFiber™ acted as an osteoconductive agent. The type II collagen
35 implant studies yielded surprising results in that 10 mg of this collagen was found to promote new bone formation in the 5-mm osteotomy model (FIG. 22A, FIG. 22B, and FIG.

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22C). New bone - bridging the osteotomy gap - was identified three weeks after surgery in 5/5 animals that received a type II collagen implant alone (i.e., minus DNA of any type). In contrast, fibrous granulation tissue, but no evidence of new bone formation, was obtained in 5/5 animals receiving a type I collagen implant alone.

Radiographic analysis demonstrated conclusively that all animals receiving an osteotomy with a type II collagen implant without exception showed radio-dense material in the osteotomy gap (FIG. 22A). In sharp contrast, radiographic analysis of all animals receiving a type I collagen implant revealed no radio-dense material forming in the osteotomy gap (FIG. 22B). The arrow in FIG. 22A point to the new bone growth formed in the osteotomy gap of type II collagen implanted-animals. No such new bone growth was observed in the animals receiving type I collagen implants (FIG. 22B).

FIG. 22C demonstrates the results of the osteotomy with a type II collagen implant. The arrow points to the area of new bone formed in the osteotomy gap. In contrast, only fibrous granulation tissue was identified in the type I collagen gap.

Previous studies have suggested that type II collagen plays only a structural role in the extracellular matrix. The results of the type II collagen implant studies are interesting because they demonstrate a novel and osteoconductive role for type II collagen during endochondral bone repair. To further optimize the osteoconductive potential of type II collagen, a yeast expression vector that encodes for type II collagen (full length $\alpha 1(\text{II})$ collagen) will be employed to produce recombinant $\alpha 1(\text{II})$ collagen protein.

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EXAMPLE XV

IDENTIFICATION OF FURTHER OSTEOTROPIC GENES:

ISOLATION OF A NOVEL LATENT TGF- β
BINDING PROTEIN-LIKE (LTBP-3) GENE

5 The TGF- β s represent a family of structurally related molecules with diverse effects on mammalian cell shape, growth, and differentiation (Roberts and Sporn, 1990). Initially synthesized as a precursor consisting of an amino-terminal propeptide followed by mature TGF- β , two chains of nascent pro-TGF- β associate in most tissues to form a Mr ~106,000 inactive disulfide-bonded dimer. Homodimers are most common, but heterodimers have also been described (Cheifetz et al., 1987; Ogawa et al., 1992). During biosynthesis the mature TGF- β dimer is cleaved from the propeptide dimer. TGF- β latency results in part from the non-covalent association of propeptide and mature TGF- β dimers (Pircher et al., 1984 and 1986; Wakefield et al., 1987; Millan et al., 1992; see also Miyazono and Heldin, 1989). Consequently, the propeptide dimer is often referred to as the latency associated protein (LAP), and LAP plus the disulfide-bonded TGF- β dimer are also known as the small latent complex. In the extracellular space small latent complexes must be dissociated to activate mature TGF- β . The mechanism of activation of the latent complex is thought to be one of the most important steps governing TGF- β effects (Lyons et al., 1988; Antonelli-Orlidge et al., 1989; Twardzik et al., 1990; Sato et al., 1993).

In certain lines of cultured cells small latent growth factor complexes may contain additional high molecular weight proteins. The best characterized of these high molecular weight proteins is the latent TGF- β binding protein, or LTBP (Miyazono et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990; Olofsson et al., 1992; Taketazu et al., 1994). LTBP produced by different cell

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types is heterogeneous in size, perhaps because of alternative splicing or because of tissue-specific proteolytic processing (Miyazono et al., 1988; Wakefield et al., 1988; Kanzaki et al., 1990; Tsuji et al., 1990).

5 Latent TGF- β complexes that contain LTBP are known as large latent complexes. LTBP has no known covalent linkage to mature TGF- β , but rather it is linked by a disulfide bond to LAP.

10 Two LBTPs have been isolated to date. The deduced human LTBP-1 amino acid sequence is comprised of a signal peptide, 16 epidermal growth factor-like repeats with the potential to bind calcium (EGF-CB repeats), 2 copies of a unique motif containing 8 cysteine residues, an RGD cell
15 attachment motif, and an 8 amino acid motif identical to the cell binding domain of the laminin B2 chain (Kanzaki et al., 1990). There is evidence that LTBP-1 binds calcium, which, in turn, induces a structural change that protects LTBP from proteolytic attack (Colosetti et al.,
20 1993). LTBP-2 shows 41% sequence identity to LTBP-1, and its structural domains show a similar overall organization (Moren et al., 1994).

While the functions of LTBP-1 and LTBP-2 presently
25 are unknown, several ideas have been put forward in the literature. First, LTBP may regulate the intracellular biosynthesis of latent TGF- β precursors. Cultured erythroleukemia cells efficiently assemble and secrete large latent TGF- β complexes, whereas they slowly secrete
30 small latent TGF- β complexes that contain anomalous disulfide bonds (Miyazono et al., 1991; Miyazono et al., 1992). Therefore, LTBP may facilitate the normal assembly and secretion of latent TGF- β complexes. Second, LTBP may target latent TGF- β to specific types of
35 connective tissue. Recent evidence suggests that the large latent TGF- β complex is covalently bound to the extracellular matrix via LTBP (Taipale et al., 1994).

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Based on these observations, LTBP has been referred to as a "matrix receptor", i.e. a secreted protein that targets and stores latent growth factors such as TGF- β to the extracellular matrix. Third, LTBP may modulate the
5 activation of latent complexes. This idea is based in part on recent evidence which suggests that mature TGF- β is released from extracellular storage sites by proteases such as plasmin and thrombin and that LTBP may protect small latent complexes from proteolytic attack (Falcone
10 et al., 1993; Benezra et al., 1993; Taipale et al., 1994), i.e. protease activity may govern the effect of TGF- β in tissues, but LTBP may modulate this activity. Fourth, LTBP may play an important role in targeting the latent TGF- β complex to the cell surface, allowing
15 latent TGF- β to be efficiently activated (Flaumenhaft et al., 1993).

A. MATERIALS AND METHODS

20 1. cDNA Cloning

Aliquots (typically 40-50,000 PFU) of phage particles from a cDNA library in the λ ZAPII[®] vector made from NIH 3T3 cell mRNA (Stratagene) and fresh overnight
25 XL1-Blue[™] cells (grown in Luria broth supplemented with 0.4% maltose in 10 mM MgSO₄) were mixed, incubated for 15 min. at 37°C, mixed again with 9 ml of liquid (50°C) top layer agarose (NZY broth plus 0.75% agarose), and then spread evenly onto freshly poured 150 mm NZY-agar plates.
30 Standard methods were used for the preparation of plaque-lifts and filter hybridization (42°C, in buffer containing 50% formamide, 5X SSPE, 1X Denhardt's, 0.1% SDS, 100 mg/ml salmon sperm DNA, 100 mg/ml heparin). Filters were washed progressively to high stringency
35 (0.1X SSC/0.1% SDS, 65°C). cDNA probes were radiolabeled by the nick translation method using commercially available reagents and protocols (Nick Translation Kit,

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Boehringer Mannheim). Purified phage clones were converted to pBluescript® plasmid clones, which were sequenced using Sequenase (v2.0) as described (Chen et al., 1993; Yin et al., 1995). Sequence alignment and identity was determined using sequence analysis programs from the Genetics Computer Group (MacVector).

2. Tissue In Situ Hybridization

To prepare normal sense and antisense probes, a unique 342 bp fragment from the 3' untranslated region (+3973 to +4314, counting the "A" of the initiator Met codon as +1; see "ish", Fig. 1) was subcloned into the pBSKS+ plasmid (Stratagene, Inc.). Template DNA was linearized with either *EcoRI* or *BamHI*, extracted, and precipitated with ethanol. Sense and antisense transcripts were generated from 1 mg template with T3 and T7 polymerases in the presence of [³⁵S]UTP at >6 mCi/ml (Amersham, >1200 Ci/mmol) and 1.6 U/ml RNasin (Promega), with the remaining *in vitro* transcription reagents provided in a kit (SureSite, Novagen Inc.). After transcription at 37°C for 1 h, DNA templates were removed by a 15 min. digest at 37°C with 0.5 U/ml RNase-free DNase I, extracted, and precipitated with ethanol. Riboprobes were hydrolyzed to an average final length of 150 bp by incubating in 40 mM NaHCO₃, 60 mM Na₂CO₃, 80 mM DTT for 40 min. at 60°C. Hydrolysis was terminated by addition of sodium acetate, pH 6.0, and glacial acetic acid to .09 M and 0.56% (v/v), respectively, and the probes were then ethanol precipitated, dissolved in 0.1 M DTT, counted, and stored at -20°C until use. Day 8.5-9.0, day 13.5, and day 16.5 mouse embryo tissue sections (Novagen) and the *in situ* hybridization protocol

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were exactly as described (Chen et al., 1993; Yin et al., 1995).

3. Northern Analysis

5 MC3T3-E1 cell poly(A+) RNA (2-10 mg aliquots) was electrophoresed on a 1.25% agarose/2.2 M formaldehyde gel and then transferred to a nylon membrane (Hybond-N, Amersham). The RNA was cross-linked to the membrane by
10 exposure to a UV light source (1.2×10^6 mJ/cm², UV Stratalinker 2400, Stratagene) and then pre-hybridized for >15 min. at 65°C in Rapid-Hyb buffer (Amersham, Inc.). A specific cDNA probe consisting solely of
15 untranscribed sequence from the 3' end of the transcript was ³²P-labeled by random priming and used for hybridization (2 h at 65°C). Blots were washed progressively to high stringency (0.1X SSC/0.1% SDS, 65°C), and then placed against x-ray film with intensifying screens (XAR, Kodak) at -86°C.

4. Antibody Preparation

LTBP-3 antibodies were raised against a unique peptide sequence found in domain #2 (amino acids
25 155-167). Peptide #274 (GESVASKHAIYAVC) (SEQ ID NO:16) was synthesized using an ABI model 431A synthesizer employing FastMoc chemistry. The sequence was confirmed using an ABI473 protein sequencer. A cysteine residue was added to the carboxy-terminus to facilitate
30 crosslinking to carrier proteins. For antibody production, the synthetic peptide was coupled to rabbit serum albumin (RSA) using MBS (*m*-maleimidobenzoic acid-*N*-hydroxysuccinimide ester) at a substitution of 7.5 mg peptide per mg of RSA. One mg of the peptide-RSA
35 conjugate in 1 ml of Freund's complete adjuvant was injected subcutaneously at 10 different sites along the backs of rabbits. Beginning at 3 weeks after initial

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immunization, the rabbits were given bi-weekly booster injections of 1 mg peptide-RSA in 100 μ l of Freund's incomplete adjuvant. IgG was prepared by mixing immune serum with caprylic acid (0.7 ml caprylic acid per ml serum), stirring for 30 min., and centrifuging at 5,000 \times g for 10 min. The supernatant was decanted and dialyzed against two changes of phosphate buffered saline (PBS) overnight at 4°C. The antibody solution was then affinity purified by passing it over a column containing the immunizing peptide coupled to Affi-gel 10 affinity support. Bound antibodies were eluted with 0.2 M glycine (pH 2.3), immediately dialyzed against PBS, and concentrated to 1 mg/ml. prior to storage at -70°C.

5. Transfection

Transient transfection was performed using standard protocols (Sambrook et al., 1989). Briefly, subconfluent cells (covering ~20% of a 100 mm plastic tissue culture dish) were washed 2x in DMEM tissue culture medium (GIBCO) and then incubated for 3 hrs. at 37°C in a sterile mixture of DEAE-dextran (0.25 mg/ml), chloroquine (55 mg/ml), and 15 mg plasmid DNA (Courey and Tjian, 1988). Cells then were shocked by incubation with 10% DMSO in sterile PBS for 2 min. at 37°C, washed 2x with DMEM (Sambrook et al., 1989), and incubated in DMEM plus 10% fetal calf serum and antibiotics for 72 hr. at 37°C.

6. Immunoprecipitation

For immunoprecipitation, 1 ml of antibody (1:400 final concentration, in PBS-TDS buffer: 0.38 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 1% Triton X-100, 0.5% Deoxycholic acid, and 0.1% SDS) was added to 1 ml of radiolabeled medium proteins. The mixture was incubated with shaking at 4°C for 1 hr., protein A-sepharose CL-4B beads were added (200 μ l, 10% suspension), and this

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mixture was incubated with shaking for one additional hour at 4°C. Immunoprecipitated proteins were pelleted by brief centrifugation, the pellet was washed 6x with PBS-TDS buffer, 2x protein loading dye was added, and the samples were boiled for 5 min. and then fractionated on 4-18% gradient SDS-PAGE (Bonadio et al., 1985). Cold molecular weight markers (200 kDa-14.3 kDa, Rainbow mix, Amersham) were used to estimate molecular weight. The gel was dried and exposed to film for the indicated time at room temperature.

7. Western Analysis

Fractionated proteins within SDS-polyacrylamide gels were transferred to a nitrocellulose filter for 2 hours using Tris-glycine-methanol buffer, pH 8.3 at 0.5 mA/cm². The filter was blocked, incubated with nonfat milk plus antibody (1:1000 dilution) for 2 hr, and washed. Antibody staining was visualized using the ECL Western blotting reagent (Amersham) according to the manufacturer's protocols.

B. RESULTS

In this study, the inventors isolated and characterized a novel murine fibrillin-like cDNA encoding LTBP-3. To clone the murine LTBP-3 gene, cDNA from a 3T3 cell cDNA library was amplified using human fibrillin-1 PCR[™] primers under low stringency conditions (i.e., annealing at 37°C initially for 10 cycles, followed by annealing at 60°C for 30 cycles). The results indicated that a murine DNA fragment of unexpectedly low homology (~50%) to human fibrillin-1 was obtained. Molecular cloning of the authentic murine fibrillin-1 transcript was also performed, confirming the human and murine fibrillin-1 coding sequences share >95% sequence identity. The murine fibrillin-1 and PCR[™] sequences were

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different, which suggested that the PCR™ product may have been derived from a related, fibrillin-like cDNA. The 3T3 cell cDNA library was screened at high stringency using the murine PCR™ product as the probe in order to test this hypothesis. A cDNA walking strategy eventually yielded seven overlapping cDNA clones (FIG. 14). It provides a unique mRNA of 4,314 nucleotides, with an open reading frame of 3,753 nucleotides (SEQ ID NO:2). The deduced molecule is a unique polypeptide of 1,251 amino acids (SEQ ID NO:3). Excluding the signal peptide (21 amino acids), the novel fibrillin-like molecule consists of five structurally distinct regions (Region 1- Region 5), and although similar to murine fibrillin-1 (FIG. 15A), its domain structure is unique as is evidenced by the schematic representation of LTBP-3 shown in FIG. 15B.

Domain #1 is a 28 amino acid segment with a net basic charge (est. pI, 12.36) that may allow for binding acidic molecules in the extracellular matrix (e.g., acidic proteoglycans). Sequences rich in basic amino acids may also function as endoproteolytic processing signals (Barr, 1991; Steiner et al., 1992), which suggests that the NH₂-terminus may be proteolytically processed. Domain #2 extends for of 390 amino acids, consisting of an EGF-like repeat, a 135 amino acid segment that was proline-rich (20.7%) and glycine-rich (11.8%) but not cysteine-rich, a Fibmotif (Pereira et al., 1993), an EGF-CB repeat, and a TGF-bp repeat. Domain #3 is a 113 amino acid segment characterized by its high proline content (21%). Domain #4 extends for 678 amino acids and consists of 14 consecutive cysteine-rich repeats. Based on structural homologies, 12/14 repeats were epidermal growth factor-calcium binding (EGF-CB) motifs (Handford et al., 1991), whereas 2/14 were transforming growth factor- β -binding protein (TGF-bp) motifs (Kanzaki et al., 1990). Finally, domain #5 is a 22 amino acid segment at the carboxy-terminus. The

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conceptual amino acid sequence encoded by the open reading frame consisted of 1,251 amino acids (FIG. 15B) with an estimated pI of 5.92, a predicted molecular mass of 134,710 Da, and five potential N-linked glycosylation sites. No RGD sequence was present.

Northern blot analysis of murine embryo RNA using a 3' untranslated region probe identified a transcript band of ~4.6 kb. In this regard, 4,310 nt have been isolated by cDNA cloning, including a 3' untranslated region of 401 nt and a 5' upstream sequence of 156 nt. The apparent discrepancy between the Northern analysis result and the cDNA sequence analysis suggested that the 5' upstream sequence may include ~300 nt of additional upstream sequence. This estimate was consistent with preliminary primer extension mapping studies indicating that the 5' upstream sequence is 400-500 nt in length.

A total of 19 cysteine-rich repeats were found in domains #2 and #4 of the murine LTBP-like (LTBP-3) polypeptide. Thirteen were EGF-like and 11/13 contained the calcium binding consensus sequence. This consensus was derived from an analysis of 154 EGF-CB repeats in 23 different proteins and from structural analyses of the EGF-CB repeat, both bound and unbound to calcium ion (Selander-Sunnerhagen et al., 1992). Variations on the consensus have been noted previously and one of these, D-L-N/D-E-C₁, was identified in the third EGF-like repeat of domain #4. In addition, a potential calcium binding sequence which has not previously been reported (E-T-N/D-E-C₁) was identified in the first EGF-like repeat of domain #4. Ten of thirteen EGF-CB repeats also contained a second consensus sequence which represents a recognition sequence for an Asp/Asn hydroxylase that co- and post-translationally modifies D/N residues (Stenflo et al., 1987; Gronke et al., 1989).

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Although about one-half the size, the deduced polypeptide was organized like fibrillin-1 in that it consisted of a signal peptide followed by 5 structurally distinct domains, i.e., two domains with numerous EGF-like, EGF-CB and Fib repeats and a third with a proline-rich sequence (Pereira et al., 1993). However, comparison of each of these domains using the GAP and BESTFIT programs (Genetics Computer Group) has revealed a low level of amino acid homology of only 27% over the five structural domains shared by the deduced murine polypeptide and human fibrillin-2. These values are low for a putative fibrillin family member because fibrillin-1 and fibrillin-2 share ~50% identity (Zhang et al., 1994).

A search of available databases revealed that the deduced murine polypeptide was most similar to the human and rat latent TGF- β binding proteins (Kanzaki et al., 1990; Tsuji et al., 1990). In this regard LTBP was found to be similar to fibrillin in that it could also be divided into five structurally distinct domains (FIG. 15A, FIG. 15B, and FIG. 15C). These include a relatively short domain downstream of the signal peptide with a net basic charge (amino acids 21-33, est. pI, 11.14); a domain consisting of EGF-like, EGF-CB, TGF-bp, and Fib motifs plus a proline-rich and glycine-rich sequence (amino acids 34-407); a proline-rich domain (amino acids 408-545); a large, domain consisting of EGF-CB, TGF-bp, and TGF-bp-like repeat motifs (amino acids 546-1379); and a relatively short domain at the carboxy terminus (amino acids 1380-1394). Amino acid sequence comparison of the deduced murine and human polypeptides shows 60% identity for domain #1, 52% identity for domain #2, 30% identity for domain #3, 43% identity for domain #4, and 7% identity for domain #5. The average identity over the five domains shared by the murine polypeptide and human

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cardiovascular tissue (myocardium plus endocardium) was also observed.

Microscopy of day 13.5 and day 16.5 embryos demonstrated expression of the murine gene by skeletal muscle cells and by cells involved in intramembranous and endochondral bone formation. The transcript was expressed by osteoblasts and by periosteal cells of the calvarium, mandible and maxilla. The transcript was also identified in both cartilage and bone of the lower extremity. A positive signal was detected in perichondrial cells and chondrocytes (proliferating > mature > hypertrophic) of articular cartilage, the presumptive growth plate, and the cartilage model within the central canal. The positive signal was also expressed by blood vessel endothelial cells within the mid-diaphysis, and the surrounding muscle cells (FIG. 18A, FIG. 18B, FIG. 18C, FIG. 18D, FIG. 18E, FIG. 18F, FIG. 18G, FIG. 18H, FIG. 18I, FIG. 18J, FIG. 18K, FIG. 18L, FIG. 18M, FIG. 18N, FIG. 18O, and FIG. 18P).

Respiratory epithelial cells lining developing small airways and connective tissue cells in the pulmonary interstitium expressed the murine transcript, as did myocardial cells (atria and ventricles) and endocardial cushion tissue. Cells within the walls of large arteries also expressed the transcript. Expression of the murine gene was identified in several organs of the alimentary system, including the tongue, esophagus, stomach, small and large intestine, pancreas and liver. Mucosal epithelial cells lining the upper and lower digestive tract plus the smooth muscle and connective tissue cells found in the submucosa expressed the transcript, as did acinar cells of the exocrine pancreas. Despite the high level of transcript expression in the liver, these results suggest both cell populations express the LTBP-3 transcript.

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In the kidney, expression above the basal level was observed in cells of developing nephrons, the ureteric bud, kidney blastema and the kidney interstitium. In the skin, epidermal and adnexal keratinocytes, dermal
5 connective tissue cells, and brown fat cells within the dorsal subcutis expressed the murine transcript. In the central and peripheral nervous systems, ganglion cells within the cerebrum, brainstem, spinal cord, and peripheral nerves expressed the murine transcript. The
10 transcript was also intensely expressed by cells of the developing murine retina.

Thus, the murine gene is widely expressed by both epithelial and connective tissue cells, a pattern that
15 would be expected for a latent TGF- β binding protein. Three final observations argue that the LTBP-like (LTBP-3) sequence presented in FIG. 25 is not simply the murine homologue of human LTBP. First, domain #4 of the murine LTBP-like (LTBP-3) sequence has a smaller number of EGF-
20 like repeat motifs than human and rat LTBP (8 versus 11). Second, portions of the human and rat LTBP-like coding sequence were characterized and found to share ~90% identity with human and rat LTBP but only 65% identity with the murine LTBP-like gene. Third, the human LTBP
25 and LTBP-like genes are localized to separate chromosomes. Human LTBP was assigned to human chromosome 2 based on the analysis of human x rodent somatic cell hybrid lines (Stenman *et al.*, 1994). The present invention represents the first mapping of an LTBP gene in
30 the murine. The human LTBP-like genes was recently localized to chromosome 11 band q12, while the murine gene was mapped to murine chromosome 19, band B (a region of conserved synteny), using several independent approaches, including fluorescent *in situ* hybridization.
35

The first indication of alternative splicing came from molecular cloning studies in the murine, in which

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independent cDNA clones were isolated with a deletion of 51 bp from the coding sequence. PCR™/Southern blot analysis provided additional evidence that the homologous 51 bp sequence was alternatively spliced in normal murine embryo tissues.

Northern blot analysis also demonstrated that the novel fibrillin gene was also expressed in rat callus three weeks after osteotomy, after mineralization has begun. Gene expression in normal adult rat bone tissue was insignificant, which suggests that microfibrils are an important part of the bone fracture healing response. The novel fibrillin-like gene was expressed in callus as a pair of alternatively spliced transcripts. This result has been independently reproduced on three occasions. Molecular cloning of the novel fibrillin gene in both murine and rat has identified potential splice junction sites for the alternative splicing event.

MC3T3-E1 murine pre-osteoblasts were used to demonstrate that the murine gene product was capable of binding TGF- β . MC3T3-E1 cells were utilized because they synthesize and secrete TGF- β , which may act as an autocrine regulator of osteoblast proliferation (Amarnani et al., 1993; Van Vlasselaer et al., 1994; Lopez-Casillas et al., 1994).

To determine whether or not MC3T3-E1 cells co-expressed the murine gene product of TGF- β , cells were plated on 100-mm dishes under differentiating conditions (Quarles et al., 1992) and the medium was replaced twice weekly. Parallel dishes were plated and assayed for cell number and alkaline phosphatase activity, which confirmed that osteoblast differentiation was indeed taking place. Equal aliquots of total cellular RNA was prepared from these MC3T3-E1 cells after 5, 14 and 28 days in culture for Northern blot analysis. As shown in FIG. 19,

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expression of the new murine gene peaked on day 14 of culture. Since MC3T3-E1 cells also show a peak in alkaline phosphatase activity on day 14 of culture (Quarles et al., 1992), the results suggest for the first time that LTBP-2 gene expression is an early marker of osteoblast differentiation.

C. DISCUSSION

This study reports the molecular cloning of a novel LTBP-like gene that contains numerous EGF-like repeats. Northern analysis indicates that the gene encodes a single transcript of ~4.6 kb in murine embryo tissues. The deduced amino acid sequence of the murine gene product appears to be a secreted polypeptide of 1,251 amino acids. Although it is similar to fibrillin, the overall structural organization and expression pattern of this gene product most resembles LTBP, a latent TGF- β binding protein that was originally isolated and characterized by Heldin and co-workers (Kanzaki et al., 1990). Several observations strongly suggest that LTBP and the murine LTBP-like gene product are therefore derived from related but distinct genetic loci. First, LTBP and the LTBP-like coding sequence share ~40% identity and differences exist in the number of EGF-CB repeats in the deduced polypeptide sequence of the two molecules. Second, a portion of the murine LTBP gene has been cloned and shown to share ~90% identity with human and rat LTBP. Conversely, portions of the human and rat LTBP-like genes have been cloned and shown to share ~90% identity with the murine LTBP-like gene. Third, LTBP and the LTBP-like gene reside on different human chromosomes (Stenman et al., 1994). Taken together, these data suggest that a family of at least two LTBP genes exists.

Similarities in the structural organization of LTBP-1 and the fibrillin-1 and fibrillin-2 polypeptides have

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been noted previously (Pereira et al., 1993; Zhang et al., 1994; Taipale et al., 1994). For example, LTBP-1 and the fibrillins are all secreted extracellular matrix constituents. Moreover, each polypeptide can be
5 organized into five domains, two of which consists predominantly of EGF-CB and TGF- β repeat motifs. LTBP-1 and fibrillin-1 also share a domain that is proline-rich, and LTBP possesses an 8-cysteine repeat previously referred to as the "Fib motif" because it was assumed to
10 be unique to fibrillin (Pereira et al., 1993). These similarities likely explain the initial isolation and cloning of the LTBP-2 PCR[™] product, especially since the human oligonucleotide primers used to initially amplify murine cDNA were designed to direct the synthesis of an
15 EGF-CB repeat in domain #4.

Another point of distinction between LTBP-2 and fibrillin concerns the spacing of conserved cysteines C₄ and C₅ in EGF-like repeats. Fibrillin-1 and fibrillin-2
20 each contain >50 such repeats, and in every one the spacing is C₄-X-C₅. While this pattern is repeated in a majority of the EGF-like repeats in LTBP-1 and LTBP-2, both genes also contain repeats with the spacing C₄-X-X-C₅. Although the significance of this observation is
25 unclear, variation in the number of amino acids between C₄ and C₅ would not be expected to alter the function of the EGF-like repeat. Mature EGF is a 48 amino acid secreted polypeptide consisting of two subdomains that have few interdomain contacts (Engel, 1989; Davis, 1990). The
30 larger NH₂-terminal subdomain consists of residues 1-32 and is stabilized by a pair of disulfide bonds (C₁-C₃ and C₂-C₄), whereas the smaller COOH-terminal subdomain (amino acids 33-48) is stabilized by a single disulfide bond (C₅-C₆). The COOH-terminal subdomain has a highly conserved
35 conformation that only is possible if certain residues and the distances between them are well conserved, while conformation-sequence requirements for the NH₂-terminal

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subdomain are relatively relaxed. Variation in C₄-C₅ spacing would not be expected to alter conformation because these residues do not normally form a disulfide bond and the spacing variation occurs at the interface of subdomains that would not be predicted to interact. The cloning of additional genes will decide whether variation in C₄-C₅ spacing is a reliable discriminator between members of the LTBP and fibrillin gene families.

10 The LTBP-2 gene is expressed more widely during development than fibrillin-1 or fibrillin-2. Studies in developing murine tissues have shown that the *Fbn-1* gene is expressed by mesenchymal cells of developing connective tissue, whereas the murine LTBP-like gene is
15 intensely expressed by epithelial, parenchymal and stromal cells. Earlier reports have suggested that TGF- β plays a role in differentiation and morphogenesis during murine development (Lyons and Moses, 1990), when TGF- β is produced by epithelial, parenchymal and stromal cells.
20 Tsuji et al., (1990) and others have suggested that the expression of TGF- β binding proteins should mirror that of TGF- β itself; the expression pattern of the LTBP-2 gene over the course of murine development is consistent with this expectation. However, the LTBP-2 gene may not
25 be completely co-regulated with TGF- β . TGF- β gene and protein expression during murine development has been surveyed extensively (Heine et al., 1987; Lehnert and Akhurst, 1988; Pelton et al., 1989; Pelton et al., 1990a,b; Millan et al., 1991); these studies have not
30 identified expression by skeletal muscle cells, chondrocytes, hepatocytes, ganglion cells, mucosal cells lining the gut, and epithelial cells of developing nephrons. It is conceivable that the LTBP-2 molecule has

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an additional function in certain connective tissues besides targeting TGF- β .

5 The binding properties of the LTBP-2 gene product are under investigation. Formally, the LTBP-2 polypeptide may bind a specific TGF- β isoform, another member of the TGF- β superfamily (e.g., a bone morphogenetic protein, inhibin, activin, or Mullerian inhibiting factor), or a growth factor unrelated to TGF-
10 β . Anti-peptide antibodies to the murine LTBP-2 polypeptide have been generated and osteoblast cell lines that express the molecule at relatively high levels have been identified. Studies with these reagents suggest that LTBP-2 assembles intracellularly into large latent
15 complexes with a growth factor that is being characterized by immunological methods.

The presence of dibasic amino acids in the LTBP-2 sequence suggests that it may undergo cell- and tissue-specific proteolysis. TGF- β regulates extracellular
20 matrix production by suppressing matrix degradation (through a decrease in the expression of proteases such as collagenase, plasminogen activator, and stromelysin plus an increase in the expression of proteinase
25 inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1) and by stimulating matrix macromolecule synthesis (for recent review, see Lyons and Moses, 1990; Massague, 1990; Laiho and Keski-Oja, 1992; Miyazono et al., 1992). Conversely,
30 production of extracellular matrix has been shown to down regulate TGF- β gene expression (Streuli et al., 1993). TGF- β may therefore regulate extracellular matrix production through a sophisticated feedback loop that influences the expression of a relatively large number of
35 genes. LTBP-1 and LTBP-2 may contribute to this regulation by facilitating the assembly and secretion of large latent growth factor complexes and then targeting

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the complex to specific connective tissues (Taipale et al., 1994).

If LTBP-3 is like LTBP-1, it has the potential to function as a secreted, extracellular structural protein. As demonstrated here, domain #1 of LTBP-3 appears to be a unique sequence that likely has a globular conformation. Domain #1 also is highly basic and may facilitate LTBP-2 binding to acidic molecules (e.g., acidic proteoglycans) within the extracellular space. Sequences rich in basic amino acids have also been shown to function as endoproteolytic processing signals for several peptide hormones (Barr, 1991; Steiner et al., 1992). It is possible, therefore, that the NH₂-terminus of LTBP-3 is proteolytically processed in a tissue-specific manner. Domains #2 and #4 consist of consecutive cysteine-rich repeats, the majority of which are of the EGF-CB type. Besides binding calcium (Corson et al., 1993), these repeats may provide LTBP-3 with regions conformation capable of interacting with other matrix macromolecules (Engel, 1989). Domain #3 is proline rich and may be capable of bending (or functioning like a hinge) in three-dimensional space (MacArthur and Thornton, 1991). (In this regard, domain #2 is of interest because it has a similar stretch of 135 amino acids that is both proline- and glycine-rich. Since glycine-rich sequences are also thought to be capable of bending or functioning like a hinge in three-dimensional space, this amino acid sequence may interrupt the extended conformation of domain #2, thereby providing it with a certain degree of flexibility in three-dimensional space.) Domain #5 also appears to be a unique sequence having a globular conformation. The absence of a known cell attachment motif may indicate that, in contrast to LTBP-1, the LTBP-3 molecule may have a more limited role in the extracellular matrix (i.e., that of a structural protein)

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in addition to its ability to target latent TGF- β complexes to specific connective tissues.

MC3T3-E1 pre-osteoblasts co-express LTBP-3 and
5 TGF- β 1 and these proteins form a complex in the culture
medium. These results are particularly interesting
because bone represents one of the largest known
repositories of latent TGF- β (200 μ g/kg bone; Seyedin
et al., 1986 and 1987), and because this growth factor
10 plays a critical role in the determination of bone
structure and function. For example, TGF- β is thought to
(i) provide a powerful stimulus to bone formation in
developing tissues, (ii) function as a possible "coupling
factor" during bone remodeling (a process that
15 coordinates bone resorption and formation), and (iii)
exert a powerful bone inductive stimulus following
fracture. Activation of the latent complex may be an
important step governing TGF- β effects, and LTBP may
modulate the activation process (e.g., it may "protect"
20 small latent complexes from proteolytic attack).

Expression of large latent TGF- β complexes bearing
LTBP may be physiologically relevant to, i.e., may be
part of the mechanism of, the pre-osteoblast \rightarrow osteoblast
25 differentiation cascade. This is based on the evidence
that MC3T3-E1 cells express large latent TGF- β 1 complexes
bearing LTBP-2 precisely at the time of transition from
the pre-osteoblast to osteoblast phenotype (~day 14 in
culture, or, at the onset of alkaline phosphatase
30 expression; see Quarles et al., 1992). The organ culture
model, for example, likely is comprised of differentiated
osteoblasts but few bone progenitors, making it a
difficult model at best in which to study the
differentiation cascade (Dallas et al., 1984). It is
35 also well known that MG63, ROS17/2.8 and UMR 106 cells
are rapidly dividing and they express the osteoblast
phenotype. Thus, these osteoblast-like cell lines do not

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show the uncoupling of cell proliferation and cell differentiation that characterizes the normal (physiologically relevant) pre-osteoblast → osteoblast transition (Gerstenfeld et al., 1984; Stein and Lian, 1993). Therefore, the production of small versus large latent TGF- β complexes may be associated with specific stages in the maturation of bone cells.

LTBP-3 may bind calcium, since EGF-CB repeats have been shown to mediate high affinity calcium binding in LTBP-1 and other proteins (Colosetti et al., 1993). Calcium binding, in turn, may contribute to molecular conformation and the regulation of its interactions with other molecules. The presence of dibasic amino acids suggests that LTBP-3 may also undergo cell- and tissue-specific proteolysis. TGF- β regulates extracellular matrix production by suppressing matrix degradation (through a decrease in the expression of proteases such as collagenase, plasminogen activator, and stromelysin plus an increase in the expression of proteinase inhibitors such as plasminogen activator inhibitor-1 and tissue inhibitor of metalloproteinase-1) and by stimulating matrix macromolecule synthesis (for recent reviews, see Lyons and Moses, 1990; Massague, 1990; Laiho and Keski-Oja, 1992; and Miyazono et al., 1993). Conversely, production of extracellular matrix has been shown to down regulate TGF- β gene expression (Streuli et al., 1993). TGF- β may therefore regulate extracellular matrix production through a sophisticated feedback loop that influences the expression of a relatively large number of genes. LTBP-1, LTBP-2, and LTBP-3 may contribute to this regulation by facilitating the assembly and secretion of large latent growth factor

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complexes and then targeting the complex to specific connective tissues (Taipale et al., 1994).

EXAMPLE XVI

5 PREPARATION OF ANTIBODIES AGAINST THE LTBP-3 GENE PRODUCT

10 An affinity-purified antibody (#274) capable of immunoprecipitating was prepared against the murine LTBP-3 gene product. A full-length murine cDNA was assembled into the pCDNA3 mammalian expression vector (Invitrogen) and expressed following transient transfection of 293T cells. Nascent polypeptides, radiolabeled by addition of ³⁵S Cys to the medium of transfected cells, were immunoprecipitated using affinity-purified antibody #274. 15 As shown in FIG. 20, the new murine polypeptide was estimated to be 180-190 kDa. To ensure the specificity of #274 binding, we showed that preincubation with 10 µg of synthetic peptide blocks immunoprecipitation of the 180-190 kDa band.

20 Finally, MC3T3-E1 cells were cultured for 7 days under differentiating conditions and double-labeled with 30 µCi/ml ³⁵S cysteine and ³⁵S methionine in deficient media. Radiolabeled media was dialyzed into cold PBS with protease inhibitors. Aliquots of the dialyzed 25 medium sample (10⁶ incorporated CPM) were analyzed by a combined immunoprecipitation/Western analysis protocol. The murine polypeptide was clearly and reproducibly secreted by MC3T3-E1 cells, migrating under reducing 30 conditions as a single band of 180-190 kDa (FIG. 21). Consistent with the results of previous studies (e.g., Miyazono et al., 1988; Dallas et al., 1994; Moren et al., 1994), bands of 70 and 50 kDa corresponding to the TGF-β1 precursor were co-immunoprecipitated with the 180 kDa 35 LTBP-3 protein. Weak bands of 40 and 12 kDa were also identified in experiments in which only immunoprecipitation was performed. The latter were not

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included in FIG. 21 because they migrated within that portion of the gel included in the Western analysis. Protein bands of 70-12.5 kDa are not variant forms of LTBP-3; FIG. 20 demonstrates that LTBP-3 migrates as a single band of 180-190 kDa following transient transfection of 293T cells, which fail to make TGF- β 1. By immunoprecipitation, a unique band consistent with monomeric mature TGF- β 1 was found in the LTBP-2 immunoprecipitate. Antibody #274 is incapable of binding TGF- β 1 as determined by radioimmunoassay using commercially available reagents (R&D Systems) and the manufacturer's suggested protocols. These results have been reproduced in 6 independent experiments which utilized 3 separate lots of MC3T3-E1 medium. Thus the new murine LTBP-3 polypeptide binds TGF- β *in vitro*.

EXAMPLE XVII

ISOLATION OF A GENE ENCODING MURINE LTBP-2

In addition to determining the DNA and corresponding polypeptide sequence of the murine LTBP-3 gene, the murine LTBP-2 gene was also cloned and sequenced.

The complete cDNA nucleotide sequence for murine LTBP-2 is shown in FIG. 27 (SEQ ID NO:17). The deduced amino acid sequence is shown in FIG. 28 (SEQ ID NO:18).

EXAMPLE XVIII

EXPRESSION OF RECOMBINANT TYPE II COLLAGEN

The *Pichia* Expression Kit (Invitrogen, Inc.) may be used to prepare recombinant type II collagen. This kit, based on the methylotrophic yeast, *Pichia pastoris*, allows high-level expression of recombinant protein in an easy-to-use relatively inexpensive system. In the absence of the preferred carbon source, glucose, *P.*

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pastoris utilizes methanol as a carbon source. The AOX1 promoter controls the gene that codes for the expression of the enzyme alcohol oxidase, which catalyzes the first step in the metabolism of methanol. This promoter, which is induced by methanol, has been characterized and incorporated into a series of *Pichia* expression vectors. This feature of *Pichia* has been exploited to express high levels of recombinant proteins often in the range of grams per liter. Because it is eukaryotic, *P. pastoris* utilizes posttranslational modification pathways that are similar to those used by mammalian cells. This implies that the recombinant type II collagen will be glycosylated and will contain disulfide bonds.

The inventors contemplate the following particular elements to be useful in the expression of recombinant type II collagen: the DNA sequence of human type II collagen (SEQ ID NO:11) (Lee et al., 1989); rat type II collagen (SEQ ID NO:13) (Michaelson, et al., 1994); and/or mouse type II collagen (SEQ ID NO:15) (Ortman, et al., 1994). As other sources of DNA sequences encoding type II collagen are available, these three are examples of many sequence elements that may be useful in the present invention.

For preparation of a recombinant type II collagen, the native type II collagen cDNA is modified by the addition of a commercially available epitope tag (the HA epitope, Pharmacia, LKB Biotechnology, Inc.). Such fragments may be readily prepared by, for example, directly synthesizing the fragment by chemical means, by application of nucleic acid reproduction technology, such as the PCR technology of U.S. Patent 4,603,102 (herein incorporated by reference) or by introducing selected sequences into recombinant vectors for recombinant production. (PCR™ is a registered trademark of Hoffmann-LaRoche, Inc.). This is followed by cloning into the

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Pichia expression vector. The resulting plasmid is characterized by DNA sequence analysis, linearized by digestion with NotI, and spheroplasts will be prepared and transformed with the linearized construct according to the manufacturer's recommendations.

Transformation facilitates a recombination event *in vivo* between the 5' and 3' AOX1 sequences in the *Pichia* vector and those in the *Pichia* genome. The result is the replacement of AOX1 with the gene of interest.

Transformants are then plated on histidine-deficient media, which will select for successfully transformed cells. Transformants are further selected against slow growth on growth media containing methanol. Positive transformants are grown for 2 days in liquid culture and then for 2-6 days in broth that uses methanol as the sole carbon source. Protein expression is evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western hybridization using a commercially available polyclonal antisera to the HA epitope (Pharmacia).

Recombinant type II collagen protein can be purified according to the manufacturer's recommendations, dialyzed against double distilled, deionized water and lyophilized in 10 mg aliquots. The aliquots are sterilized and used as implant material for the osteoconductive matrices.

30

* * *

All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations

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may be applied to the composition, methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be

5 apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and

10 modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5 (i) APPLICANT:

(A) NAME: REGENTS OF THE UNIVERSITY OF MICHIGAN

(B) STREET: 3003 S. State Street

The Wolverine Tower, Room 2071

(C) CITY: Ann Arbor

10 (D) STATE: Michigan

(E) COUNTRY: United States of America

(F) POSTAL (ZIP) CODE: 48109-1280

15 (ii) INVENTORS: BONADIO, Jeffrey

ROESSLER, Blake J.

GOLDSTEIN, Steven A.

LIN, Wushan

20 (iii) TITLE OF INVENTION: METHODS AND COMPOSITIONS

FOR STIMULATING BONE CELLS

(iv) NUMBER OF SEQUENCES: 18

(v) CORRESPONDENCE ADDRESS:

25 (A) ADDRESSEE: Arnold, White & Durkee

(B) STREET: P.O. Box 4433

(C) CITY: Houston

(D) STATE: Texas

(E) COUNTRY: United States of America

30 (F) ZIP: 77210

(vi) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

35 (C) OPERATING SYSTEM: PC-DOS/MS-DOS/ASCII

(D) SOFTWARE: PatentIn Release #1.0, Version
#1.30

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(vii) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: UNKNOWN
- (B) FILING DATE: CONCURRENTLY HERewith
- (C) CLASSIFICATION: UNKNOWN

5

(viii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/316,650
- (B) FILING DATE: 30-SEP-1994
- (C) CLASSIFICATION: UNKNOWN

10

- (A) APPLICATION NUMBER: US 08/199,780
- (B) FILING DATE: 18-FEB-1994
- (C) CLASSIFICATION: UNKNOWN

15

(ix) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Parker, David L.
- (B) REGISTRATION NUMBER: 32,165
- (C) REFERENCE/DOCKET NUMBER: UMIC009P--

20

(x) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (512) 418-3000
- (B) TELEFAX: (713) 789-2679
- (C) TELEX: 79-0924

25

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 417 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: protein

-164-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Met Ile Pro Gly Asn Arg Met Leu Met Val Val Leu Leu Cys Gln Val
 1 5 10 15

5

Leu Leu Gly Gly Ala Thr Asp Ala Ser Leu Met Pro Glu Thr Gly Lys
 20 25 30

Lys Lys Val Ala Glu Ile Gln Gly His Ala Gly Gly Arg Arg Ser Gly
 35 40 45

10

Gln Ser His Glu Leu Leu Arg Asp Phe Glu Ala Thr Leu Leu Gln Met
 50 55 60

Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys Ser Ala Val Ile Pro
 65 70 75 80

Asp Tyr Met Ser Asp Leu Tyr Arg Leu Gln Ser Gly Glu Glu Glu
 85 90 95

20

Glu Glu Gln Ser Gln Gly Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala
 100 105 110

-165-

Ser Ser Ala Asn Thr Val Ser Ser Phe His His Glu Glu His Leu Glu
 115 120 125
 Asn Ile Pro Gly Thr Ser Glu Ser Ser Ala Phe Arg Phe Phe Asn
 130 135 140
 Leu Ser Ser Ile Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg
 145 150 155 160
 Leu Phe Arg Glu Gln Val Asp Gln Gly Pro Asp Trp Glu Gln Gly Phe
 165 170 175
 His Arg Met Asn Ile Tyr Glu Val Met Lys Pro Pro Ala Glu Met Val
 180 185 190
 Pro Gly His Leu Ile Thr Arg Leu Leu Asp Thr Ser Leu Val Arg His
 195 200 205
 Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro Ala Val Leu Arg
 210 215 220
 Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu Val Thr
 225 230 235 240

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His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Ser Ile Ser
245 250 255

Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu
260 265 270

Leu Val Thr Phe Gly His Asp Gly Arg Gly His Thr Leu Thr Arg Arg
275 280 285

Ser Ala Lys Arg Ser Pro Lys His His Pro Gln Arg Ser Ser Lys Lys
290 295 300

Asn Lys Asn Cys Arg Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val
305 310 315 320

Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr Gln Ala Phe Tyr
325 330 335

Cys His Gly Asp Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr
340 345 350

Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Ser Ile
355 360 365

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-167-

Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser Met Leu
 370 375 380
 Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu Met
 385 390 395 400
 Val Val Glu Gly Cys Gly Cys Arg Tyr Pro Tyr Asp Val Pro Asp Tyr
 405 410 415

10 Ala

(2) INFORMATION FOR SEQ ID NO:2:

- 15 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3753 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
- 20 (ii) MOLECULE TYPE: DNA (genomic)

-168-

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..3753

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATG CGC CAG GCC GCA TTG GGG CTG CTG GCA CTA CTC CTG CTG GCG CTG 48

Met Arg Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Ala Leu

1

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CTG GGC CCC GGC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGG GCA CAG 96

Leu Gly Pro Gly Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala Gln

20

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30

15 GCG GGG GCG GGC CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT 144

Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val Val Phe Ala Pro

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GTG ATC TGC AAG CCG ACC TGT CTG AAG GGC CAG TGT CGG GAC AGC TGT 192

20 Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys

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 Gln Gln Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly His Ser Thr
 65 70 75 80

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 Asp Thr Leu Thr Gly Ser Ala Phe Arg Val Val Cys Pro Leu Pro
 85 90 95

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 10 Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu Cys Pro
 100 105 110

CCG GAT TTC ACG GGG CGC TTC TGC CAG GTG CCT GCT GCA GGA ACC GGA 384
 Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly
 115 120 125

15 GCT GGC ACC GGG AGT TCA GGC CCC GGC TGG CCC GAC CGG GCC ATG TCC 432
 Ala Gly Thr Gly Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser
 130 135 140

20 ACA GGC CCG CTG CCG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC 480
 Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu Gly Glu Ser Val Ala Ser
 145 150 155 160

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528
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 Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro
 165 170 175

576
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 Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly
 180 185 190

624
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 Pro Gly Gln Ile Ser Ala Glu Val Gln Ala Pro Pro Val Val Asn
 195 200 205

672
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 Val Arg Val His His Pro Pro Glu Ala Ser Val Gln Val His Arg Ile
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720
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 225 230 235 240

768
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 His Pro Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly
 245 250 255

-171-

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 Arg Cys Phe Gln Asp Thr Leu Pro Lys Gln Pro Cys Gly Ser Asn Pro
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 Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile Gly Thr
 275 280 285

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 Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr
 290 295 300

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 325 330 335

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 Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn
 340 345 350

-172-

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 Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro
 355 360 365

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10 TTC CGC CTT GTG AGC ACC GAA CAC CAG TGC CAG CAC CCT CTG ACC ACA 1200
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 Ala Arg Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu
 420 425 430

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 Ile Cys Pro Gly Trp Glu Arg Val Pro Tyr Pro His Leu Pro Pro Asp
 435 440 445

-173-

1392
 GCT CAC CAT CCA GGG GGA AAG CGA CTT CTC CCT CTT CCT GCA CCC GAC
 Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala Pro Asp
 450 455 460

1440
 5 GGG CCA CCC AAA CCC CAG CAG CTT CCT GAA AGC CCC AGC CGA GCA CCA
 Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro
 465 470 475 480

1488
 10 CCC CTC GAG GAC ACA GAG GAG GAA GAG AGA GGA GTG ACC ATG GAT CCA CCA
 Pro Leu Glu Asp Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro
 485 490 495

1536
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 Val Ser Glu Glu Arg Ser Val Gln Gln Ser His Pro Thr Thr Thr Thr
 500 505 510

1584
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 Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro
 515 520 525

1632
 20 ACC TTC CAC CGG TTC CTG CCA GAC TTG CCC CCA TCC CGA AGT GCA GTG
 Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val
 530 535 540

-174-

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	545 550 555 560	
5	CAG AAT ATC TGT GGC CAT GGA CAG TGT GTG CCT GGC CCC TCG GAT TAC	1728
	Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly Pro Ser Asp Tyr	
	565 570 575	
10	TCC TGC CAC TGC AAC GCT GGC TAC CGG TCA CAC CCG CAG CAC CGC TAC	1776
	Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr	
	580 585 590	
15	TGT GTT GAT GTG AAC GAG TGC GAG GCA GAG CCC TGC GGC CCC GGG AAA	1824
	Cys Val Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys	
	595 600 605	
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	610 615 620	
	GGC TAC CGC CTC CAC GTG GGT GCA GGG GGC CGC TCG TGC GTG GAC CTG	1920
	Gly Tyr Arg Leu His Val Gly Ala Gly Gly Arg Ser Cys Val Asp Leu	
	625 630 635 640	

-175-

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 Asn Phe Pro Gly His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu 660 665 670

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 Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp Ile Asp Glu Cys Arg Asp 675 680 685

 CCT AGC ACC TGC CCT GAT GGC AAA TGT GAA AAC AAA CCT GGC AGC TTC 2112
 Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe 690 695 700

 AAG TGC ATC GCC TGC CAG CCT GGC TAC CGT AGC CAG GGG GGC GCC 2160
 Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Ala 705 710 715 720

 TGT CGT GAT GTC AAC GAA TGC TCC TCC GAA GGT ACC CCC TGC TCT CCT GGA 2208
 Cys Arg Asp Val Asn Glu Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly 725 730 735

-176-

	TGG TGT GAG AAA CTT CCG GGT TCT TAC CGT TGC ACG TGT GCC CAG GGG	2256
	Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr Cys Ala Gln Gly	
	740 745 750	
5	ATA CGA ACC CGC ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT	2304
	Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Asp Cys	
	755 760 765	
	GAG GCT GGG AAA GTG TGC CAA GAT GGC ATC TGC ACG AAC ACA CCA GGC	2352
10	Glu Ala Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly	
	770 775 780	
	TCT TTC CAG TGT CAG TGC CTC TCC GGC TAT CAT CTG TCA AGG GAT CGG	2400
	Ser Phe Gln Cys Gln Cys Leu Ser Gly Tyr His Leu Ser Arg Asp Arg	
15	785 790 795 800	
	AGC CGC TGT GAG GAC ATT GAT GAA TGT GAC TTC CCT GCG GCC TGC ATC	2448
	Ser Arg Cys Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala Cys Ile	
	805 810 815	
20	GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA TGT CTC TGT CCC	2496
	Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro	
	820 825 830	

-177-

	CTG GGT CAT CGG TTG GTG GGC AGG AAG TGC AAG AAA GAT ATA GAT	2544
	Leu Gly His Arg Leu Val Gly Gly Arg Lys Cys Lys Lys Asp Ile Asp	
	835 840 845	
5	GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC	2592
	Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu Pro His Ala Cys Glu Asn	
	850 855 860	
	CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC	2640
10	Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr	
	865 870 875 880	
	CAG GAC CAG CAT GGG TGT GAG GAG GTG GAG CAG CCC CAC CAC AAG AAG	2688
	Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys	
15	885 890 895	
	GAG TGC TAC CTT AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG	2736
	Glu Cys Tyr Leu Asn Phe Asp Asp Thr Val Phe Cys Asp Ser Val Leu	
	900 905 910	
20	GCT ACC AAT GTC ACT CAG CAG GAA TGC TGT TGC TCT CTG GGA GCT GGC	2784
	Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Ser Leu Gly Ala Gly	
	915 920 925	

-178-

TGG GGA GAC CAC TGC GAA ATC TAT CCC TGT CCA GTC TAC AGC TCA GCC 2832
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 930 935 940

5 GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA AGG CTA CAC TCA GGA CAA 2880
 Glu Phe His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln
 945 950 955 960

CAA CAT TGT GAA CTA TGC ATC CCT GCC CAC CGT GAC ATC GAC GAA TGC 2928
 10 Gln His Cys Glu Leu Cys Ile Pro Ala His Arg Asp Ile Asp Glu Cys
 965 970 975

ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG AAC TCG 2976
 Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val Asn Ser
 980 985 990

CAG CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC TAC GAT GGC 3024
 Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly
 995 1000 1005

AAC CTG CTG GAG TGC GTG GAC GTG GAC GAG TGC TTG GAT GAG TCT AAC 3072
 Asn Leu Leu Glu Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn
 1010 1015 1020

-179-

TGC AGG AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC 3120
 Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys
 1025 1030 1035 1040

5 ACT CCG CCG GCA GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG ATC CCG 3168
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 1045 1050 1055

GAG AGA TGG AGC ACG CCC CAG AGA GAC GTG AAG TGT GCT GGG GCC AGC 3216
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 1060 1065 1070

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 1075 1080 1085

15 ACT TTT GAT GAC TGC TGC TGC CGC CAG CCG CGG CTG GGT ACC CAG TGC 3312
 Thr Phe Asp Asp Cys Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys
 1090 1095 1100

20 AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC CCG ACT TCA CAG 3360
 Arg Pro Cys Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln
 1105 1110 1115 1120

-180-

AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG GGG AAG 3408
Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Gly Lys
1125 1130 1135

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Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg
1140 1145 1150

TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GGC GTA TGC GAG 3504
Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Ala Val Cys Glu
1155 1160 1165

TGT CCT GGA GGC TTT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC 3552
Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp
1170 1175 1180

15 ATT GAT GAG TGC CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC 3600
Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser
1185 1190 1195 1200

20 GAG CCG TGC GTG AAC ACC AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT 3648
Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala
1205 1210 1215

-181-

GGC TTC ACG CGC AGC CGC CCT CAC GGG CCT GCG TGC CTC AGC GCC GCC 3696
Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala
1220 1225 1230

5 GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA GTG ATC GAT CAT CGA GGG 3744
Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly
1235 1240 1245

TAT TTT CAC 3753
10 Tyr Phe His
1250

(2) INFORMATION FOR SEQ ID NO:3:

15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1251 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

-182-

Met Arg Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Ala Leu
 1 5 10 15
 Leu Gly Pro Gly Arg Gly Val Gly Arg Pro Gly Ser Gly Ala Gln
 5 20 25 30
 Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val Val Phe Ala Pro
 35 40 45
 Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys
 10 50 55 60
 Gln Gln Gly Ser Asn Met Thr Leu Ile Gly Glu Asn Gly His Ser Thr
 65 70 75 80
 Asp Thr Leu Thr Gly Ser Ala Phe Arg Val Val Cys Pro Leu Pro
 85 90 95
 Cys Met Asn Gly Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu Cys Pro
 20 100 105 110
 Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly
 115 120 125

-183-

Ala Gly Thr Gly Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser
 130 135 140

Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu Gly Glu Ser Val Ala Ser
 5 145 150 155 160

Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro
 165 170 175

10 Gly Glu Gly Pro Pro Ala Gln His Ala Ala Phe Leu Val Pro Leu Gly
 180 185 190

Pro Gly Gln Ile Ser Ala Glu Val Gln Ala Pro Pro Pro Val Val Asn
 195 200 205

15 Val Arg Val His His Pro Pro Glu Ala Ser Val Gln Val His Arg Ile
 210 215 220

Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro
 20 225 230 235 240

His Pro Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly
 245 250 255

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Arg Cys Phe Gln Asp Thr Leu Pro Lys Gln Pro Cys Gly Ser Asn Pro
260 265 270

Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile Gly Thr
275 280 285

5

Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr
290 295 300

Gly Val Gln Lys Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys
305 310 315 320

Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr His Cys Gln Asp Ile Asn
325 330 335

15

Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn
340 345 350

Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro
355 360 365

20

Leu Ala Ala Gln Cys Ile Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys
370 375 380

-185-

Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His Pro Leu Thr Thr
 385 390 395 400

Arg Leu Thr Arg Gln Leu Cys Cys Ser Val Gly Lys Ala Trp Gly
 405 410 415

5

Ala Arg Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu
 420 425 430

Ile Cys Pro Gly Trp Glu Arg Val Pro Tyr Pro His Leu Pro Pro Asp
 435 440 445

Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala Pro Asp
 450 455 460

15

Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro
 465 470 475 480

Pro Leu Glu Asp Thr Glu Glu Glu Arg Gly Val Thr Met Asp Pro Pro
 485 490 495

20

Val Ser Glu Glu Arg Ser Val Gln Gln Ser His Pro Thr Thr Thr Thr
 500 505 510

- 186 -

Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro
 515 520 525
 Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val
 530 535 540
 Glu Ile Ala Pro Thr Gln Val Thr Glu Thr Asp Glu Cys Arg Leu Asn
 545 550 555 560
 Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly Pro Ser Asp Tyr
 565 570 575
 Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr
 580 585 590
 Cys Val Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys
 595 600 605
 Gly Ile Cys Met Asn Thr Gly Gly Ser Tyr Asn Cys His Cys Asn Arg
 610 615 620
 Gly Tyr Arg Leu His Val Gly Ala Gly Gly Arg Ser Cys Val Asp Leu
 625 630 635 640

-187-

Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile
645 650 655

5 Asn Phe Pro Gly His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu
660 665 670

Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp Ile Asp Glu Cys Arg Asp
675 680 685

10 Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe
690 695 700

Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Ala
705 710 715 720

15 Cys Arg Asp Val Asn Glu Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly
725 730 735

20 Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr Cys Ala Gln Gly
740 745 750

Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Asp Cys
755 760 765

-188-

Glu Ala Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly
 770 775 780
 Ser Phe Gln Cys Gln Cys Leu Ser Gly Tyr His Leu Ser Arg Asp Arg
 5 785 790 795 800
 Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala Cys Ile
 805 810 815
 10 Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro
 820 825 830
 Leu Gly His Arg Leu Val Gly Gly Arg Lys Cys Lys Asp Ile Asp
 835 840 845
 15 Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu Pro His Ala Cys Glu Asn
 850 855 860
 Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr
 20 865 870 875 880
 Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys
 885 890 895

-189-

Glu Cys Tyr Leu Asn Phe Asp Asp Thr Val Phe Cys Asp Ser Val Leu
 900 910
 Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Ser Leu Gly Ala Gly
 915 920 925
 Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala
 930 935 940
 Glu Phe His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln
 945 950 955 960
 Gln His Cys Glu Leu Cys Ile Pro Ala His Arg Asp Ile Asp Glu Cys
 965 970 975
 Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val Asn Ser
 980 985 990
 Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly
 995 1000 1005
 Asn Leu Leu Glu Cys Val Asp Val Asp Glu Cys Leu Asp Glu Ser Asn
 1010 1015 1020

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Cys Arg Asn Gly Val Cys Glu Asn Thr Trp Arg Leu Pro Cys Ala Cys
 1025 1030 1035 1040
 Thr Pro Pro Ala Glu Tyr Ser Pro Ala Gln Ala Gln Cys Leu Ile Pro
 1045 1050 1055
 Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser
 1060 1065 1070
 Glu Glu Arg Thr Ala Cys Val Trp Gly Pro Trp Ala Gly Pro Ala Leu
 1075 1080 1085
 Thr Phe Asp Asp Cys Cys Arg Gln Pro Arg Leu Gly Thr Gln Cys
 1090 1100
 Arg Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln
 1105 1110 1115 1120
 Ser Glu Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Gly Lys
 1125 1130 1135
 Ser Pro Arg Asp Glu Asp Ser Ser Glu Glu Asp Ser Asp Glu Cys Arg
 1140 1145 1150

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Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Gly Ala Val Cys Glu
 1155 1160 1165

Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp
 1170 1175 1180

Ile Asp Glu Cys Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser
 1185 1190 1195 1200

10 Glu Arg Cys Val Asn Thr Ser Gly Ser Phe Arg Cys Val Cys Lys Ala
 1205 1210 1215

Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala
 1220 1225 1230

15 Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly
 1235 1240 1245

Tyr Phe His
 20 1250

(2) INFORMATION FOR SEQ ID NO:4:

- 192 -

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

10

AACATGACGC TCATCGGAGA GAAC

24

(2) INFORMATION FOR SEQ ID NO:5:

15

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

25 AGGTGATCGC AGATCCTC

18

(2) INFORMATION FOR SEQ ID NO:6:

30

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: DNA (genomic)

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TACCGATGCT ACCGCAGCAA TCTT

24

5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

10

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATGCCTAAAC TCTACCAGCA CG

22

20

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

25

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAGTCACGTC ATCCATTCCA CA

22

35

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGTCCAAGTT GTGTCTTAGC AG

22

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 53 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

-195-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gly Pro Pro Gly Pro Gln Gly Ala Thr Gly Pro Leu Gly Pro Lys Gly
 1 5 10 15
 Gln Thr Gly Glu Pro Gly Ile Ala Gly Phe Lys Gly Glu Gln Gly Pro
 20 25 30
 Lys Gly Glu Thr Gly Pro Ala Gly Pro Gln Gly Ala Pro Gly Pro Ala
 35 40 45
 Gly Glu Glu Gly Lys
 50

15 (2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 159 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

-196-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCCCTCCCG GTCCCTAAGG TGCAACTGGT CCTCTGGGCC CCAAGGTCA GACGGGTGAG 60
CCCCGGCATCG CTGGCTTCAA AGGTGAACAA GGGCCCAAGG GAGAGACTGG ACCTGCTGGG 120
CCCCAGGGAG CCCCTGGCCC TGCTGGTGAA GAAGGAAAA 159

10 (2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1442 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

-197-

Met Ile Arg Leu Gly Ala Pro Gln Ser Leu Val Leu Leu Thr Leu Leu
 1 5 10 15

Ile Ala Ala Val Leu Arg Cys Gln Gly Gln Asp Ala Gln Glu Ala Gly
 20 25 30 35

Ser Cys¹Leu Gln Asn Gly Gln Arg Tyr Lys Asp Lys Asp Val Trp Lys
 35 40 45

Pro Ser Ser Cys Arg Ile Cys Val Cys Asp Thr Gly Asn Val Leu Cys
 50 55 60

Asp Asp Ile Ile Cys Glu Asp Pro Asp Cys Leu Asn Pro Glu Ile Pro
 65 70 75 80

Phe Gly Glu Cys Cys Pro Ile Cys Pro Ala Asp Leu Ala Thr Ala Ser
 85 90 95

Gly Arg Lys Leu Gly Pro Lys Gly Gln Lys Gly Glu Pro Gly Asp Ile
 100 105 110

Arg Asp Gly Pro Ala Gly Glu Gln Gly Pro Arg Gly Asp Arg Gly Asp
 115 120 125

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Lys Gly Glu Lys Asn Phe Ala Ala Gln Met Ala Gly Gly Tyr Asp Glu
 130 135 140
 Lys Ala Gly Gly Ala Gln Met Gly Val Met Gln Gly Pro Met Gly Pro
 145 150 155 160
 Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Ala Pro Gly Pro Gln
 165 170 175
 Gly Phe Gln Gly Asn Pro Gly Glu Pro Gly Glu Pro Gly Val Ser Gly
 180 185 190
 Pro Met Gly Pro Arg Gly Pro Pro Gly Pro Ala Gly Lys Pro Gly Asp
 195 200 205
 Asp Gly Glu Ala Gly Lys Pro Gly Lys Ser Gly Glu Arg Gly Leu Pro
 210 215 220
 Gly Pro Met Gly Ala Arg Gly Phe Pro Gly Thr Pro Gly Leu Pro Gly
 225 230 235 240
 Val Lys Gly His Arg Gly Tyr Pro Gly Leu Asp Gly Ala Lys Gly Glu
 245 250 255

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Ala Gly Ala Pro Gly Val Lys Gly Glu Ser Gly Ser Pro Gly Glu Asn
 260 265 270

Gly Ser Pro Gly Pro Met Gly Pro Arg Gly Leu Pro Gly Glu Arg Gly
 275 280 285

Arg Thr Gly Pro Ala Gly Ala Ala Gly Ala Arg Gly Asn Asp Gly Gln
 290 295 300

Pro Gly Pro Ala Gly Pro Pro Gly Pro Val Gly Pro Ala Gly Gly Pro
 305 310 315 320

Gly Phe Pro Gly Ala Pro Gly Ala Lys Gly Glu Ala Gly Pro Thr Gly
 325 330 335

Ala Arg Gly Pro Glu Gly Ala Gln Gly Ser Arg Gly Glu Pro Gly Asn
 340 345 350

Pro Gly Ser Pro Gly Pro Ala Gly Ala Ser Gly Asn Pro Gly Thr Asp
 355 360 365

Gly Ile Pro Gly Ala Lys Gly Ser Ala Gly Ala Pro Gly Ile Ala Gly
 370 375 380

-200-

Ala Pro Gly Phe Pro Gly Pro Arg Gly Pro Pro Gly Pro Gln Gly Ala
385 390 395 400

Thr Gly Pro Leu Gly Pro Lys Gly Gln Ala Gly Glu Pro Gly Ile Ala
405 410 415

5

Gly Phe Lys Gly Asp Gln Gly Pro Lys Gly Glu Thr Gly Pro Ala Gly
420 425 430

Pro Gln Gly Ala Pro Gly Pro Ala Gly Glu Glu Gly Lys Arg Gly Ala
435 440 445

10

Arg Gly Glu Pro Gly Gly Ala Gly Pro Ile Gly Pro Pro Gly Glu Arg
450 455 460

15

Gly Ala Pro Gly Asn Arg Gly Phe Pro Gly Gln Asp Gly Leu Ala Gly
465 470 475 480

Pro Lys Gly Ala Pro Gly Glu Arg Gly Pro Ser Gly Leu Ala Gly Pro
485 490 495

20

Lys Gly Ala Asn Gly Asp Pro Gly Arg Pro Gly Glu Pro Gly Leu Pro
500 505 510

-201-

Gly Ala Arg Gly Leu Thr Gly Arg Pro Gly Asp Ala Gly Pro Gln Gly
515 520 525

Lys Val Gly Pro Ser Gly Ala Pro Gly Glu Asp Gly Arg Pro Gly Pro
530 535 540

Pro Gly Pro Gln Gly Ala Arg Gly Gln Pro Gly Val Met Gly Phe Pro
545 550 555 560

Gly Pro Lys Gly Ala Asn Gly Glu Pro Gly Lys Ala Gly Glu Lys Gly
565 570 575

Leu Ala Gly Ala Pro Gly Leu Arg Gly Leu Pro Gly Lys Asp Gly Glu
580 585 590

Thr Gly Ala Ala Gly Pro Pro Gly Pro Ser Gly Pro Ala Gly Glu Arg
595 600 605

Gly Glu Gln Gly Ala Pro Gly Pro Ser Gly Phe Gln Gly Leu Pro Gly
610 615 620

Pro Pro Gly Pro Pro Gly Glu Gly Lys Gln Gly Asp Gln Gly Ile
625 630 635 640

-202-

Pro Gly Glu Ala Gly Ala Pro Gly Leu Val Gly Pro Arg Gly Glu Arg
645 650 655

Gly Phe Pro Gly Glu Arg Gly Ser Pro Gly Ala Gln Gly Leu Gln Gly
660 665 670

Pro Arg Gly Leu Pro Gly Thr Pro Gly Thr Asp Gly Pro Lys Gly Ala
675 680 685

Ala Gly Pro Asp Gly Pro Pro Gly Ala Gln Gly Pro Pro Gly Leu Gln
690 695 700

Gly Met Pro Gly Glu Arg Gly Ala Ala Gly Ile Ala Gly Pro Lys Gly
705 710 715 720

Asp Arg Gly Asp Val Gly Glu Lys Gly Pro Glu Gly Ala Pro Gly Lys
725 730 735

Asp Gly Gly Arg Gly Leu Thr Gly Pro Ile Gly Pro Pro Gly Pro Ala
740 745 750

Gly Ala Asn Gly Glu Lys Gly Glu Ala Gly Pro Pro Gly Pro Ser Gly
755 760 765

5

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-203-

Ser Thr Gly Ala Arg Gly Ala Pro Gly Glu Pro Gly Glu Thr Gly Pro
770 775 780

Pro Gly Pro Ala Gly Phe Ala Gly Pro Pro Gly Ala Asp Gly Gln Pro
785 790 795 800

Gly Ala Lys Gly Asp Gln Gly Glu Ala Gly Gln Lys Gly Asp Ala Gly
805 810 815

Ala Pro Gly Pro Gln Gly Pro Ser Gly Ala Pro Gly Pro Gln Gly Pro
820 825 830

Thr Gly Val Thr Gly Pro Lys Gly Ala Arg Gly Ala Gln Gly Pro Pro
835 840 845

Gly Ala Thr Gly Phe Pro Gly Ala Ala Gly Arg Val Gly Pro Pro Gly
850 855 860

Ala Asn Gly Asn Pro Gly Pro Ala Gly Pro Pro Gly Pro Ala Gly Lys
865 870 875 880

Asp Gly Pro Lys Gly Val Arg Gly Asp Ser Gly Pro Pro Gly Arg Ala
885 890 895

-204-

Gly Asp Pro Gly Leu Glu Gly Pro Ala Gly Ala Pro Gly Glu Lys Gly
900 905 910

Glu Pro Gly Asp Asp Gly Pro Ser Gly Leu Asp Gly Pro Pro Gly Pro
915 920 925

Gln Gly Leu Ala Gly Gln Arg Gly Ile Val Gly Leu Pro Gly Gln Arg
930 935 940

Gly Glu Arg Gly Phe Pro Gly Leu Pro Gly Pro Ser Gly Glu Pro Gly
945 950 955 960

Lys Gln Gly Ala Pro Gly Ala Ser Gly Asp Arg Gly Pro Pro Gly Pro
965 970 975

Val Gly Pro Pro Gly Leu Thr Gly Pro Ala Gly Glu Pro Gly Arg Glu
980 985 990

Gly Ser Pro Gly Ala Asp Gly Pro Pro Gly Arg Asp Gly Ala Ala Gly
995 1000 1005

Val Lys Gly Asp Arg Gly Glu Thr Gly Ala Leu Gly Ala Pro Gly Ala
1010 1015 1020

-205-

Pro Gly Pro Pro Gly Ser Pro Gly Pro Ala Gly Pro Thr Gly Lys Gln
1025 1030 1035 1040

Gly Asp Arg Gly Glu Ala Gly Ala Gln Gly Pro Met Gly Pro Ser Gly
1045 1050 1055

5

Pro Ala Gly Ala Arg Gly Ile Ala Gly Pro Gln Gly Pro Arg Gly Asp
1060 1065 1070

Lys Gly Glu Ser Gly Glu Gln Gly Glu Arg Gly Leu Lys Gly His Arg
1075 1080 1085

Gly Phe Thr Gly Leu Gln Gly Leu Pro Gly Pro Pro Gly Pro Ser Gly
1090 1095 1100

15

Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly Pro Arg Gly Pro
1105 1110 1115 1120

Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ser Asn Gly Ile Pro
1125 1130 1135

20

Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser Gly Glu Thr Gly
1140 1145 1150

-206-

Pro Val Gly Pro Pro Gly Ser Pro Gly Pro Pro Gly Pro Pro Gly Pro
 1155 1160 1165

Pro Gly Pro Gly Ile Asp Met Ser Ala Phe Ala Gly Leu Gly Gln Arg
 1170 1175 1180

Glu Lys Gly Pro Asp Pro Met Gln Tyr Met Arg Ala Asp Glu Ala Asp
 1185 1190 1195 1200

Ser Thr Leu Arg Gln His Asp Val Glu Val Asp Ala Thr Leu Lys Ser
 1205 1210 1215

Leu Asn Asn Gln Ile Glu Ser Ile Arg Ser Pro Asp Gly Ser Arg Lys
 1220 1225 1230

Asn Pro Ala Arg Thr Cys Gln Asp Leu Lys Leu Cys His Pro Glu Trp
 1235 1240 1245

Lys Ser Gly Asp Tyr Trp Ile Asp Pro Asn Gln Gly Cys Thr Leu Asp
 1250 1255 1260

Ala Met Lys Val Phe Cys Asn Met Glu Thr Gly Glu Thr Cys Val Tyr
 1265 1270 1275 1280

-207-

Pro Asn Pro Ala Thr Val Pro Arg Lys Asn Trp Trp Ser Ser Lys Ser
1285 1290 1295

Lys Glu Lys Lys His Ile Trp Phe Gly Glu Thr Met Asn Gly Gly Phe
1300 1305 1310

His Phe Ser Tyr Gly Asp Gly Asn Leu Ala Pro Asn Thr Ala Asn Val
1315 1320 1325

Gln Met Thr Phe Leu Arg Leu Leu Ser Thr Glu Gly Ser Gln Asn Ile
1330 1335 1340

Thr Tyr His Cys Lys Asn Ser Ile Ala Tyr Leu Asp Glu Ala Ala Gly
1345 1350 1355 1360

Asn Leu Lys Lys Ala Leu Leu Ile Gln Gly Ser Asn Asp Val Glu Met
1365 1370 1375

Arg Ala Glu Gly Asn Ser Arg Phe Thr Tyr Thr Ala Leu Lys Asp Gly
1380 1385 1390

Cys Thr Lys His Thr Gly Lys Trp Gly Lys Thr Val Ile Glu Tyr Arg
1395 1400 1405

5

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15

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-208-

Ser Gln Lys Thr Ser Arg Leu Pro Ile Ile Asp Ile Ala Pro Met Asp
1410 1415 1420

Ile Gly Gly Ala Glu Gln Glu Phe Gly Val Asp Ile Gly Pro Val Cys
1425 1430 1435 1440

Phe Leu

10 (2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 267 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

20

-209-

ATAGGCCCTT TGGAGACGGC TGTTTTCCAG ACTCCAAACT ATCGTGTAC ACGTGTGGGA 60
AATGAAGTGT CTTTCAATTG TGAGCAAACC CTGGACCACA ATACTATGTA CTGGTACAAG 120
5 CAAGACTCTA AGAATTGCT GAAGATTATG TTAGCTTACA ATAATAAGCA ACTCATTGTA 180
AAGAAACAG TTCCAAGGCG CTTCTCACCT CAGTCTTCAG ATAAAGCTCA TTTGAATCTT 240
CGAATCAAGT CTGTAGAGCT GGAGGAC 267

10

(2) INFORMATION FOR SEQ ID NO:14:

(1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 54 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: peptide

20

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:14:

-210-

Gly Pro Ser Gly Asp Gln Gly Ala Ser Gly Pro Ala Gly Pro Ser Gly
1 5 10 15

Pro Arg Gly Pro Pro Gly Pro Val Gly Pro Ser Gly Lys Asp Gly Ala
20 25 30

Asn Gly Ile Pro Gly Pro Ile Gly Pro Pro Gly Pro Arg Gly Arg Ser
35 40 45

10 Gly Glu Thr Gly Pro Ala
50

(2) INFORMATION FOR SEQ ID NO:15:

15 (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 731 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: DNA (genomic)

-211-

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:15:

	AGAATATAGA TAGATATGTC TGTGCTGACC GTGGCCTTTT GCCTCTTCCT TCTACACAGG	60
5	GTCCTTCTGG AGACCAAGGT GCTTCTGGTC CTGCTGGTCC TTCTGGCCCT AGAGTAAAGTG	120
	ACATGGAGTT GGAAGATGGA GGGGGCCCTT CAGAGAGTGT GGGCTGTGT TCCCATGGGG	180
	AGGGAATGC TGCTGCTTCT GGGGAAGCTG TGGGCTCAGG GGTCTCACT CAGTAATGGG	240
10	GGCAGGACTG GTCATGTGC CTATGGCCAG AAAAGGCGCT GAGGCCACAA TGGCTGTAAG	300
	ACAAACATGA ATCAGCCTCT CGCTGTCAGA CAGAACAGCA TTTTACAAAG AGGAGCTTAG	360
15	GAGGGTAGGC AAGCCATGGA GCTATCCTGC TGGTTCCTGG CCAAATAGAG ACCAACTTAG	420
	GGTTCCATGA CTGAGCATGT GAAGAACTGG GGGCGGAGTG GCTGGTGCTA TCAGGACAGC	480
	CACCTACCCA GCCCCAGCGA CTCCCCAGCC TTCCCTGTGG TGACCACTCT TTCTCTACGA	540
20	CCTCTCTCTC TTGCAGGGTC CTCCTGGCCC CGTCGGTCCC TCTGGCAAAG ATGGTGCTAA	600
	TGGAATCCCT GGCCCCATTG GGCCTCCTGG TCCCCGTGGA CGATCAGCG AAACCGGCCC	660

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TGCTGTAAGT GTCTGACTC CTTCCCTGCT GTCGAGGTGT CCCTACCATC CGGAGGCTT 720
GAGCTCTTTT T 731

5 (2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: protein

15

(x1) SEQUENCE DESCRIPTION: SEQ ID NO:16:

20 Gly Glu Ser Val Ala Ser Lys His Ala Ile Tyr Ala Val Cys
1 5 10

(2) INFORMATION FOR SEQ ID NO:17:

-213-

(1) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5502 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "DNA"

10

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..5502

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

ATG GAG AGC ACC TCC CCG CGA GGT CTC CGG TGC CCA CAG CTC TGC AGC 48
Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser
1255 1260 1265

20

-214-

96
 CAC TCT GGC GCC ATG AGA GCG CCG ACC ACC GCT CGC TGC TCC GGA TGC
 His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys
 1270 1275 1280

5 144
 ATC CAA CCG GTG CGT TGG AGG GGC TTC CTG CCA CTT GTC CTG GTC GTC
 Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val
 1285 1290 1295

10 192
 TTG ATG GGG ACA AGT CAT GCC CAA CCG GAT TCC ATA GGG AGA TAC GAA
 Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu
 1300 1305 1310 1315

15 240
 CCA GCT AGC AGG GAT GCG AAT CCG TTG TGG CAC CCC GTG GGC AGC CAC
 Pro Ala Ser Arg Asp Ala Asn Arg Leu Trp His Pro Val Gly Ser His
 1320 1325 1330

20 288
 CCC GCA GCG GCT GCA GCC AAG GTG TAC AGT CTG TTC CGA GAG CCT GAC
 Pro Ala Ala Ala Ala Lys Val Tyr Ser Leu Phe Arg Glu Pro Asp
 1335 1340 1345

336
 GCG CCG GTC CCC GGC TTG TCG CCC TCT GAG TGG AAC CAG CCG GCC CAG
 Ala Pro Val Pro Gly Leu Ser Pro Ser Glu Trp Asn Gln Pro Ala Gln
 1350 1355 1360

-215-

GGG AAC CCG GGA TGG CTC GCA GAG GCC GAG GCC AGG AGG CCA CCT CGA 384
 Gly Asn Pro Gly Trp Leu Ala Glu Ala Glu Ala Arg Arg Pro Pro Arg
 1365 1370 1375

5 ACC CAG CAG CTG CGT CGA GTC CAG CCA CCT GTC CAG ACT CGG AGA AGC 432
 Thr Gln Gln Leu Arg Arg Val Gln Pro Pro Val Gln Thr Arg Arg Ser
 1380 1385 1390 1395

CAT CCC CGG GGC CAG CAG CAG ATA GCA GCC CGG GCT GCA CCT TCT GTC 480
 His Pro Arg Gly Gln Gln Gln Ile Ala Ala Arg Ala Ala Pro Ser Val
 1400 1405 1410

GCG CGC CTG GAA ACC CCT CAG CAG CGA CCC GCG GCT GCA CGG CGA GGG CGG 528
 Ala Arg Leu Glu Thr Pro Gln Arg Pro Ala Ala Ala Arg Arg Gly Arg
 1415 1420 1425

15 CTC ACT GGG AGA AAT GTC TGC GGG GGA CAG TGC TGC CCA GGA TGG ACA 576
 Leu Thr Gly Arg Asn Val Cys Gly Gly Gln Cys Cys Pro Gly Trp Thr
 1430 1435 1440

20 ACA TCA AAC AGC ACC AAC CAC TGT ATC AAA CCT GTG TGT CAG CCT CCC 624
 Thr Ser Asn Ser Thr Asn His Cys Ile Lys Pro Val Cys Gln Pro Pro
 1445 1450 1455

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TGT CAG AAC CGA GGC TCC TGC AGC AGG CCC CAG GTC TGC ATC TGC CGT 672
 Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg
 1460 1465 1470 1475

 5 TCT GGC TTC CGT GGG GCG CGC TGT GAG GAG GTC ATC CCT GAG GAG GAA 720
 Ser Gly Phe Arg Gly Ala Arg Cys Glu Val Ile Pro Glu Glu Glu
 1480 1485 1490

 TTT GAC CCT CAG AAT GCC AGG CCT GTG CCC AGA CGC TCA GTG GAG AGA 768
 10 Phe Asp Pro Gln Asn Ala Arg Pro Val Pro Arg Arg Ser Val Glu Arg
 1495 1500 1505

 GCA CCC GGT CCT CAC AGA AGC AGT GAG GCC AGA GGA AGT CTA GTG ACC 816
 Ala Pro Gly Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val Thr
 1510 1515 1520
 15
 AGA ATA CAG CCG CTG GTA CCA CCA CCA TCA CCA CCT CCA TCT CGG CGC 864
 Arg Ile Gln Pro Leu Val Pro Pro Pro Ser Pro Pro Pro Ser Arg Arg
 1525 1530 1535
 20 CTC AGC CAG CCC TGG CCC CTG CAG CAG CAC TCA GGG CCG TCC AGG ACA 912
 Leu Ser Gln Pro Trp Pro Leu Gln Gln His Ser Gly Pro Ser Arg Thr
 1540 1545 1550 1555

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GTT CGT CGG TAT CCG GCC ACT GGT GCC AAT GGC CAG CTG ATG TCC AAC 960
 Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn
 1560 1565 1570

5 GCT TTG CCT TCA GGA CTC GAG CTG AGA GAC AGC AGC CCA CAG GCA GCA 1008
 Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala
 1575 1580 1585

CAT GTG AAC CAT CTC TCA CCC CCC TGG GGG CTG AAC CTC ACC GAG AAA 1056
 10 His Val Asn His Leu Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys
 1590 1595 1600

ATC AAG AAA ATC AAA GTC GTC TTC ACC CCC ACC ATC TGC AAG CAG ACC 1104
 Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr
 1605 1610 1615

TGT GCC CGG GGA CGC TGT GCC AAC AGC TGT GAG AAG GGT GAC ACC ACC 1152
 Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr
 1620 1625 1630 1635

20 ACC TTG TAC AGT CAG GGT GGC CAT GGG CAT GAC CCC AAG TCT GGC TTC 1200
 Thr Leu Tyr Ser Gln Gly Gly His Gly His Asp Pro Lys Ser Gly Phe
 1640 1645 1650

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CGT ATC TAT TTC TGC CAA ATC CCC TGC CTG AAT GGT GGC CGC TGC ATC 1248
 Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile
 1655 1660 1665

5 GGC CGG GAC GAG TGC TGG TGT CCA GCC AAC TCC ACA GGA AAG TTC TGC 1296
 Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys
 1670 1675 1680

CAT CTG CCT GTC CCG CAG CCA GAC AGG GAA CCT GCA GGG CGA GGT TCC 1344
 10 His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser
 1685 1690 1695

CGG CAC AGA ACC CTG CTG GAA GGT CCC CTG AAG CAA TCC ACC TTC ACG 1392
 Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr
 15 1700 1705 1710 1715

CTG CCT CTC TCT AAC CAG CTC GCC TCT GTG AAC CCC TCG CTG GTG AAG 1440
 Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys
 1720 1725 1730

20 GTG CAA ATT CAT CAC CCG CCT GAG GCC TCT GTG CAG ATT CAC CAG GTG 1488
 Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val
 1735 1740 1745

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GCC CGG GTC CGG GGT GAG CTG GAG CCC GTG CTG GAG GAC AAC AGT GTG 1536
 Ala Arg Val Arg Gly Glu Leu Asp Pro Val Leu Glu Asp Asn Ser Val
 1750 1755 1760

5 GAG ACC AGA GCC TCT CAT CGC CCC CAC GGC AAC CTA GGC CAC AGC CCC 1584
 Glu Thr Arg Ala Ser His Arg Pro His Gly Asn Leu Gly His Ser Pro
 1765 1770 1775

TGG GCC AGC AAC AGC ATA CCC GCT CGG GCC GGA GAG GCC CCT CGG CCA 1632
 10 Trp Ala Ser Asn Ser Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro
 1780 1785 1790 1795

CCA CCA GTG CTG TCT AGG CAT TAT GGA CTT CTG GGC CAG TGT TAC CTG 1680
 Pro Pro Val Leu Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu
 1800 1805 1810

AGC ACG GTG AAT GGA CAG TGT GCT AAC CCC CTA GGT AGT CTG ACT TCT 1728
 Ser Thr Val Asn Gly Gln Cys Ala Asn Pro Leu Gly Ser Leu Thr Ser
 1815 1820 1825

20 CAG GAG GAC TGC TGT GGC AGT GTG GGC ACC TTC TGG GGC GTG ACC TCC 1776
 Gln Glu Asp Cys Cys Gly Ser Val Gly Thr Phe Trp Gly Val Thr Ser
 1830 1835 1840

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TGT GCT CCC TGC CCA CCC AGA CAA GAG GGT CCA GCC TTC CCA GTG ATT 1824
 Cys Ala Pro Cys Pro Pro Arg Gln Glu Gly Pro Ala Phe Pro Val Ile
 1845 1850 1855

5 GAA AAT GGC CAG CTG GAG TGT CCC CAA GGA TAC AAG AGA CTG AAC CTC 1872
 Glu Asn Gly Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg Leu Asn Leu
 1860 1865 1870 1875

AGC CAC TGC CAA GAT ATC AAT GAG TGC CTG ACC CTG GGC CTC TGC AAG 1920
 10 Ser His Cys Gln Asp Ile Asn Glu Cys Leu Thr Leu Gly Leu Cys Lys
 1880 1885 1890

GAC TCG GAG TGC GTG AAC ACC AGG GGC AGC TAC CTG TGC ACC TGC AGG 1968
 Asp Ser Glu Cys Val Asn Thr Arg Gly Ser Tyr Leu Cys Thr Cys Arg
 1895 1900 1905

15 CCT GGC CTC ATG CTG GAT CCG TCA AGG AGC CGC TGC GTA TCG GAC AAG 2016
 Pro Gly Leu Met Leu Asp Pro Ser Arg Ser Arg Cys Val Ser Asp Lys
 1910 1915 1920

20 GCT GTC TCC ATG CAG CAG GGA CTA TGC TAC CGG TCA CTG GGG TCT GGT 2064
 Ala Val Ser Met Gln Gln Gly Leu Cys Tyr Arg Ser Leu Gly Ser Gly
 1925 1930 1935

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2112
 ACC TGC ACC CTG CCT TTG GTT CAT CGG ATC ACC AAG CAG ATA TGC TGC
 Thr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys
 1940 1945 1950 1955

5 2160
 TGC AGC CGT GTG GGC AAA GCC TGG GGT AGC ACA TGT GAA CAG TGT CCC
 Cys Ser Arg Val Gly Lys Ala Trp Gly Ser Thr Cys Glu Gln Cys Pro
 1960 1965 1970

10 2208
 CTG CCT GGC ACA GAA GCC TTC AGG GAG ATC TGC CCT GCT GGC CAT GGC
 Leu Pro Gly Thr Glu Ala Phe Arg Glu Ile Cys Pro Ala Gly His Gly
 1975 1980 1985

15 2256
 TAC ACC TAC TCG AGC TCA GAC ATC CGC CTG TCT ATG AGG AAA GCC GAA
 Tyr Thr Tyr Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu
 1990 1995 2000

20 2304
 GAA GAG GAA CTG GCT AGC CCC TTA AGG GAG CAG ACA GAG CAG AGC ACT
 Glu Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr
 2005 2010 2015

20 2352
 GCA CCC CCA CCT GGG CAA GCA GAG AGG CAA CCA CTC CGG GCA GCC ACC
 Ala Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr
 2020 2025 2030 2035

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GCC ACC TGG ATT GAG GCT GAG ACC CTC CCT GAC AAA GGT GAC TCT CGG 2400
 Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg 2040
 2045 2050

5. GCT GTT CAG ATC ACA ACC AGT GCT CCC CAC CTA CCT GCC CGG GTA CCA 2448
 Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro 2055
 2060 2065

GGG GAT GCC ACT GGA AGA CCA GCA CCA TCC TTG CCT GGA CAG GGC ATT 2496
 Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile 2070
 2075 2080

CCA GAG AGT CCA GCA GAA GAG CAA GTG ATT CCC TCC AGT GAT GTC TTG 2544
 Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser Asp Val Leu 2085
 2090 2095

GTG ACA CAC AGC CCC CCA GAC TTT GAT CCA TGT TTT GCT GGA GCC TCC 2592
 Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser 2100
 2105 2110 2115

AAC ATC TGT GGC CCT GGG ACC TGT GTG AGC CTC CCA AAT GGA TAC AGA 2640
 Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg 2120
 2125 2130

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TGT GTC TGC AGC CCT GGC TAC CAG CTA CAC CCC AGC CAA GAC TAC TGT 2688
 Cys Val Cys Ser Pro Gly Tyr Gln His Pro Ser Gln Asp Tyr Cys
 2135 2140 2145

5 ACT GAT GAC AAC GAG TGT ATG AGG AAC CCC TGT GAA GGA AGA GGG CGC 2736
 Thr Asp Asp Asn Glu Cys Met Arg Asn Pro Cys Glu Gly Arg Gly Arg
 2150 2155 2160

TGT GTC AAC AGT GTG GGC TCC TAC TCC TGC CTC TGC TAT CCT GGC TAC 2784
 Cys Val Asn Ser Val Gly Ser Tyr Ser Cys Leu Cys Tyr Pro Gly Tyr
 2165 2170 2175

ACA CTA GTC ACC CTC GGA GAC ACA CAG GAG TGC CAA GAT ATC GAT GAG 2832
 Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu
 2180 2185 2190 2195

TGT GAG CAG CCC GGG GTG TGC AGT GGT GGG CGA TGC AGC AAC ACG GAG 2880
 Cys Glu Gln Pro Gly Val Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu
 2200 2205 2210

20 GGC TCG TAC CAC TGC GAG TGT GAT CGG GGC TAC ATC ATG GTC AGG AAA 2928
 Gly Ser Tyr His Cys Glu Cys Asp Arg Gly Tyr Ile Met Val Arg Lys
 2215 2220 2225

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GGA CAC TGT CAA GAT ATC AAC GAA TGC CGT CAC CCT GGT ACC TGC CCT 2976
 Gly His Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro
 2230 2235 2240

5 GAT GGG AGA TGC GTC AAC TCC CCT GGC TCC TAC ACT TGT CTG GCC TGT 3024
 Asp Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys Leu Ala Cys
 2245 2250 2255

GAG GAG GGC TAT GTA GGC CAG AGT GGG AGC TGT GTA GAT GTC AAT GAG 3072
 Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu
 2260 2265 2270 2275

TGT CTG ACC CCT GGG ATA TGT ACC CAT GGA AGG TGC ATC AAC ATG GAA 3120
 Cys Leu Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu
 2280 2285 2290

GGC TCC TTT AGA TGC TCC TGT GAG CCG GGC TAT GAG GTC ACC CCA GAC 3168
 Gly Ser Phe Arg Cys Ser Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp
 2295 2300 2305

AAG AAG GGC TGC CGA GAT GTG GAC GAG TGT GCC AGC CGA GCC TCG TGC 3216
 Lys Lys Gly Cys Arg Asp Val Asp Glu Cys Ala Ser Arg Ala Ser Cys
 2310 2315 2320

20

-225-

CCC ACG GGC CTC TGC CTC AAC ACG GAG GGC TCC TTC ACC TGC TCA GCC 3264
 Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala
 2325 2330 2335

5 TGT CAG AGC GGG TAC TGG GTG AAC GAA GAT GGC ACT GCC TGT GAA GAC 3312
 Cys Gln Ser Gly Tyr Trp Val Asn Glu Asp Gly Thr Ala Cys Glu Asp
 2340 2345 2350 2355

TTG GAT GAA TGT GCC TTC CCT GGA GTC TGC CCC ACA GGC GTC TGC ACC 3360
 10 Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr
 2360 2365 2370

AAT ACT GTA GGC TCC TTC TCC TGC AAG GAC TGT GAC CAG GGC TAC CGG 3408
 Asn Thr Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg
 15 2375 2380 2385

CCC AAC CCC CTG GGC AAC AGA TGC GAA GAT GTG GAT GAG TGT GAA GGT 3456
 Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly
 2390 2395 2400

CCC CAA AGC AGC TGC CGG GGA GGC GAA TGC AAG AAC ACA GAA GGT TCC 3504
 Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser
 2405 2410 2415

20

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TAC CAA TGC CTC TGT CAC CAG GGC TTC CAG CTG GTC AAT GGC ACC ATG 3552
 Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met
 2420 2425 2430 2435

5 TGT GAG GAC GTG AAT GAG TGT GTT GGG GAA GAG CAT TGT GCT CCT CAC 3600
 Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu His Cys Ala Pro His
 2440 2445 2450

GGC GAG TGC CTC AAC AGC CTG GGC TCC TTC TGC CTC TGT GCA CCC 3648
 Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro
 2455 2460 2465

GGC TTT GCT AGT GCT GAG GGC ACC AGA TGC CAG GAT GTT GAT GAA 3696
 Gly Phe Ala Ser Ala Glu Gly Gly Thr Arg Cys Gln Asp Val Asp Glu
 2470 2475 2480

15 TGT GCA GCC ACA GAC CCG TGT CCG GGA GGA CAC TGT GTC AAC ACA GAG 3744
 Cys Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu
 2485 2490 2495

20 GGC TCC TTC AGC TGT CTG TGT GAG ACT GCT TCC TTC CAG CCC TCC CCA 3792
 Gly Ser Phe Ser Cys Leu Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro
 2500 2505 2510 2515

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GAC AGC GGA GAA TGT TTG GAT ATT GAT GAG TGT GAG GAC CGT GAA GAC 3840
 Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp 2520 2525 2530

5 CCG GTG TGC GGA GCC TGG AGG TGT GAG AAC AGT CCT GGT TCC TAC CGC 3888
 Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg 2535 2540 2545

TGC ATC CTG GAC TGC CAG CCT GGA TTC TAT GTG GCG CCA AAT GGA GAC 3936
 10 Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp 2550 2555 2560

TGC ATT GAC ATA GAT GAA TGT GCC AAT GAC ACT GTG TGT GGG AAC CAT 3984
 Cys Ile Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His 2565 2570 2575

15 GGC TTC TGT GAC AAC ACG GAC GGC TCC TTC CGC TGC CTG TGT GAC CAG 4032
 Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln 2580 2585 2590

20 GGC TTC GAG ACC TCA CCA TCA GGC TGG GAG TGT GTT GAT GTG AAC GAG 4080
 Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu 2600 2605 2610

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TGT GAG CTC ATG ATG GCA GTG TGT GGG GAT GCG CTC TGT GAG AAC GTG 4128
 Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val
 2615 2620 2625

5 GAA GGC TCC TTC CTG TGC CTT TGC GCC AGT GAC CTT GAG GAG TAC GAC 4176
 Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp
 2630 2635 2640

GCA GAA GAA GGA CAC TGC CGT CCT CGG GTG GCT GGA GCT CAG AGA ATC 4224
 10 Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile
 2645 2650 2655

CCA GAG GTC CGG ACA GAG GAC CAG GCT CCA AGC CTT ATC CGC ATG GAA 4272
 Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu
 15 2660 2665 2670 2675

TGC TAC TCT GAA CAC AAT GGT GGT CCT CCC TGC TCT CAA ATC CTG GGC 4320
 Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly
 2680 2685 2690

CAG AAC TCC ACA CAG GCC GAG TGC TGC TGC ACT CAG GGT GCC AGA TGG 4368
 Gln Asn Ser Thr Gln Ala Glu Cys Cys Cys Thr Gln Gly Ala Arg Trp
 2695 2700 2705

20

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4416
 GGA AAG GCC TGT GCG CCC TGC CCA TCT GAG GAC TCA GTT GAA TTC AGT
 Gly Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe Ser
 2710 2715 2720

4464
 5 CAG CTC TGC CCC AGT GGT CAA GGT TAC ATC CCA GTG GAA GGA GCC TGG
 Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp
 2725 2730 2735

4512
 10 ACA TTT GGA CAA ACC ATG TAT ACA GAT GCC GAT GAA TGT GTA CTG TTT
 Thr Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu Phe
 2740 2745 2750 2755

4560
 15 GGG CCT GCT CTC TGC CAG AAT GGC CGA TGC TCA AAC ATA GTG CCT GGC
 Gly Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly
 2760 2765 2770

4608
 TAC ATT TGC CTG TGC AAC CCT GGC TAC CAC TAT GAT GCC TCC AGC AGG
 Tyr Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg
 2775 2780 2785

4656
 20 AAG TGC CAG GAT CAC AAC GAA TGC CAG GAC TGT GCC TGT GAG AAC GGT
 Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly
 2790 2795 2800

-230-

GAG TGT GTG AAC CAA GAA GGC TCC TTC CAT TGC CTC TGC AAT CCC CCC 4704
 Glu Cys Val Asn Gln Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro
 2805 2810 2815

5 CTC ACC CTA GAC CTC AGT GGG CAG CGC TGT GTG AAC ACG ACC AGC AGC 4752
 Leu Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr Ser Ser
 2820 2825 2830 2835

ACG GAG GAC TTC CCT GAC CAT GAC ATC CAC ATG GAC ATC TGC TGG AAA 4800
 Thr Glu Asp Phe Pro Asp His Asp Ile His Met Asp Ile Cys Trp Lys
 2840 2845 2850

AAA GTC ACC AAT GAT GTG TGC AGC CAG CCC TTG CGT GGG CAC CAT ACC 4848
 Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr
 2855 2860 2865

ACC TAT ACA GAA TGC TGC TGC CAA GAT GGG GAG GCC TGG AGC CAG CAA 4896
 Thr Tyr Thr Glu Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln
 2870 2875 2880

20 TGC GCT CTG TGC CCG CCC AGG AGC TCT GAG GTC TAC GCT CAG CTG TGC 4944
 Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys
 2885 2890 2895

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AAC GTG GCT CGG ATT GAG GCA GAG CGC GGA GCA GGG ATC CAC TTC CGG 4992
 Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg
 2900 2905 2910 2915

 5 CCA GGC TAT GAG TAT GGC CCT GGC CTG GAC GAT CTG CCT GAA AAC CTC 5040
 Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu
 2920 2925 2930

 TAC GGC CCA GAT GGG GCT CCC TTC TAT AAC TAC CTA GGC CCC GAG GAC 5088
 Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp
 2935 2940 2945

 ACT GCC CCT GAG CCT CCC TTC TCC AAC CCA GCC AGC CAG CCG GGA GAC 5136
 Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp
 2950 2955 2960

 AAC ACA CCT GTC CTT GAG CCT CCT CTG CAG CCC TCT GAA CTT CAG CCT 5184
 Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro
 2965 2970 2975

 20 CAC TAT CTA GCC AGC CAC TCA GAA CCC CCT GCC TCC TTC GAA GGC CTT 5232
 His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu
 2980 2985 2990 2995

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CAG GCT GAG GAA TGT GGC ATC CTG AAT GGC TGT GAG AAT GGC CGC TGC 5280
 Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys
 3000 3005 3010

5 GTG CGT GTG CGG GAG GGC TAC ACT TGC GAC TGC TTT GAG GGC TTC CAG 5328
 Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln
 3015 3020 3025

CTG GAT GCG CCC ACA TTG GCC TGT GTG GAT GTG AAC GAG TGT GAA GAC 5376
 10 Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp
 3030 3035 3040

TTG AAC GGG CCT GCA CGA CTC TGT GCA CAC GGT CAC TGT GAG AAC ACA 5424
 Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr
 3045 3050 3055

GAG GGT TCC TAT CGC TGC CAC TGT TCG CCA GGT TAC GTG GCA GAG CCA 5472
 Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro
 3060 3065 3070 3075

20 GGC CCC CCA CAC TGT GCG GCC AAG GAG TAG 5502
 Gly Pro Pro His Cys Ala Ala Lys Glu *
 3080 3085

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Pro Ala Ser Arg Asp Ala Asn Arg Leu Trp His Pro Val Gly Ser His
 65 70 75 80

Pro Ala Ala Ala Ala Lys Val Tyr Ser Leu Phe Arg Glu Pro Asp
 85 90 95

5

Ala Pro Val Pro Gly Leu Ser Pro Ser Glu Trp Asn Gln Pro Ala Gln
 100 105 110

10 Gly Asn Pro Gly Trp Leu Ala Glu Ala Glu Ala Arg Arg Pro Pro Arg
 115 120 125

Thr Gln Gln Leu Arg Arg Val Gln Pro Pro Val Gln Thr Arg Arg Ser
 130 135 140

15

His Pro Arg Gly Gln Gln Gln Ile Ala Ala Arg Ala Ala Pro Ser Val
 145 150 155 160

Ala Arg Leu Glu Thr Pro Gln Arg Pro Ala Ala Ala Arg Arg Gly Arg
 165 170 175

20

Leu Thr Gly Arg Asn Val Cys Gly Gly Gln Cys Cys Pro Gly Trp Thr
 180 185 190

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(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1834 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Met Glu Ser Thr Ser Pro Arg Gly Leu Arg Cys Pro Gln Leu Cys Ser
1 5 10 15
His Ser Gly Ala Met Arg Ala Pro Thr Thr Ala Arg Cys Ser Gly Cys
20 25 30
Ile Gln Arg Val Arg Trp Arg Gly Phe Leu Pro Leu Val Leu Ala Val
35 40 45
Leu Met Gly Thr Ser His Ala Gln Arg Asp Ser Ile Gly Arg Tyr Glu
50 55 60

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Thr Ser Asn Ser Thr Asn His Cys Ile Lys Pro Val Cys Gln Pro Pro
 195 200 205
 Cys Gln Asn Arg Gly Ser Cys Ser Arg Pro Gln Val Cys Ile Cys Arg
 5 210 215 220
 Ser Gly Phe Arg Gly Ala Arg Cys Glu Glu Val Ile Pro Glu Glu Glu
 225 230 235 240
 10 Phe Asp Pro Gln Asn Ala Arg Pro Val Pro Arg Arg Ser Val Glu Arg
 245 250 255
 Ala Pro Gly Pro His Arg Ser Ser Glu Ala Arg Gly Ser Leu Val Thr
 260 265 270
 15 Arg Ile Gln Pro Leu Val Pro Pro Pro Ser Pro Pro Pro Ser Arg Arg
 275 280 285
 Leu Ser Gln Pro Trp Pro Leu Gln Gln His Ser Gly Pro Ser Arg Thr
 290 295 300
 Val Arg Arg Tyr Pro Ala Thr Gly Ala Asn Gly Gln Leu Met Ser Asn
 305 310 315 320

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Ala Leu Pro Ser Gly Leu Glu Leu Arg Asp Ser Ser Pro Gln Ala Ala
 325 330 335

His Val Asn His Leu Ser Pro Pro Trp Gly Leu Asn Leu Thr Glu Lys
 340 345 350

Ile Lys Lys Ile Lys Val Val Phe Thr Pro Thr Ile Cys Lys Gln Thr
 355 360 365

10 Cys Ala Arg Gly Arg Cys Ala Asn Ser Cys Glu Lys Gly Asp Thr Thr
 370 375 380

Thr Leu Tyr Ser Gln Gly Gly His Gly His Asp Pro Lys Ser Gly Phe
 385 390 395 400

15 Arg Ile Tyr Phe Cys Gln Ile Pro Cys Leu Asn Gly Gly Arg Cys Ile
 405 410 415

Gly Arg Asp Glu Cys Trp Cys Pro Ala Asn Ser Thr Gly Lys Phe Cys
 420 425 430

His Leu Pro Val Pro Gln Pro Asp Arg Glu Pro Ala Gly Arg Gly Ser
 435 440 445

20

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Arg His Arg Thr Leu Leu Glu Gly Pro Leu Lys Gln Ser Thr Phe Thr
 450 455 460
 Leu Pro Leu Ser Asn Gln Leu Ala Ser Val Asn Pro Ser Leu Val Lys
 5 465 470 475 480
 Val Gln Ile His His Pro Pro Glu Ala Ser Val Gln Ile His Gln Val
 485 490 495
 10 Ala Arg Val Arg Gly Glu Leu Asp Pro Val Leu Glu Asp Asn Ser Val
 500 505 510
 Glu Thr Arg Ala Ser His Arg Pro His Gly Asn Leu Gly His Ser Pro
 515 520 525
 15 Trp Ala Ser Asn Ser Ile Pro Ala Arg Ala Gly Glu Ala Pro Arg Pro
 530 535 540
 Pro Pro Val Leu Ser Arg His Tyr Gly Leu Leu Gly Gln Cys Tyr Leu
 20 545 550 555 560
 Ser Thr Val Asn Gly Gln Cys Ala Asn Pro Leu Gly Ser Leu Thr Ser
 565 570 575

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Gln Glu Asp Cys Cys Gly Ser Val Gly Thr Phe Trp Gly Val Thr Ser
580 585 590

Cys Ala Pro Cys Pro Pro Arg Gln Glu Gly Pro Ala Phe Pro Val Ile
595 600 605

Glu Asn Gly Gln Leu Glu Cys Pro Gln Gly Tyr Lys Arg Leu Asn Leu
610 615 620

10 Ser His Cys Gln Asp Ile Asn Glu Cys Leu Thr Leu Gly Leu Cys Lys
625 630 635 640

Asp Ser Glu Cys Val Asn Thr Arg Gly Ser Tyr Leu Cys Thr Cys Arg
645 650 655

15 Pro Gly Leu Met Leu Asp Pro Ser Arg Ser Arg Cys Val Ser Asp Lys
660 665 670

20 Ala Val Ser Met Gln Gln Gly Leu Cys Tyr Arg Ser Leu Gly Ser Gly
675 680 685

Thr Cys Thr Leu Pro Leu Val His Arg Ile Thr Lys Gln Ile Cys Cys
690 695 700

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Cys Ser Arg Val Gly Lys Ala Trp Gly Ser Thr Cys Glu Gln Cys Pro
 705 710 715 720
 Leu Pro Gly Thr Glu Ala Phe Arg Glu Ile Cys Pro Ala Gly His Gly
 725 730 735
 Tyr Thr Tyr Ser Ser Ser Asp Ile Arg Leu Ser Met Arg Lys Ala Glu
 740 745 750
 Glu Glu Glu Leu Ala Ser Pro Leu Arg Glu Gln Thr Glu Gln Ser Thr
 755 760 765
 Ala Pro Pro Pro Gly Gln Ala Glu Arg Gln Pro Leu Arg Ala Ala Thr
 770 775 780
 Ala Thr Trp Ile Glu Ala Glu Thr Leu Pro Asp Lys Gly Asp Ser Arg
 785 790 795 800
 Ala Val Gln Ile Thr Thr Ser Ala Pro His Leu Pro Ala Arg Val Pro
 805 810 815
 Gly Asp Ala Thr Gly Arg Pro Ala Pro Ser Leu Pro Gly Gln Gly Ile
 820 825 830

-240-

Pro Glu Ser Pro Ala Glu Glu Gln Val Ile Pro Ser Ser Asp Val Leu
835 840 845

Val Thr His Ser Pro Pro Asp Phe Asp Pro Cys Phe Ala Gly Ala Ser
850 855 860

Asn Ile Cys Gly Pro Gly Thr Cys Val Ser Leu Pro Asn Gly Tyr Arg
865 870 875 880

10 Cys Val Cys Ser Pro Gly Tyr Gln Leu His Pro Ser Gln Asp Tyr Cys
885 890 895

Thr Asp Asp Asn Glu Cys Met Arg Asn Pro Cys Glu Gly Arg Gly Arg
900 905 910

15 Cys Val Asn Ser Val Gly Ser Tyr Ser Cys Leu Cys Tyr Pro Gly Tyr
915 920 925

20 Thr Leu Val Thr Leu Gly Asp Thr Gln Glu Cys Gln Asp Ile Asp Glu
930 935 940

Cys Glu Gln Pro Gly Val Cys Ser Gly Gly Arg Cys Ser Asn Thr Glu
945 950 955 960

-241-

Gly Ser Tyr His Cys Glu Cys Asp Arg Gly Tyr Ile Met Val Arg Lys
 965 970 975

Gly His Cys Gln Asp Ile Asn Glu Cys Arg His Pro Gly Thr Cys Pro
 980 985 990

Asp Gly Arg Cys Val Asn Ser Pro Gly Ser Tyr Thr Cys Leu Ala Cys
 995 1000 1005

Glu Glu Gly Tyr Val Gly Gln Ser Gly Ser Cys Val Asp Val Asn Glu
 1010 1015 1020

Cys Leu Thr Pro Gly Ile Cys Thr His Gly Arg Cys Ile Asn Met Glu
 1025 1030 1035 1040

Gly Ser Phe Arg Cys Ser Cys Glu Pro Gly Tyr Glu Val Thr Pro Asp
 1045 1050 1055

Lys Lys Gly Cys Arg Asp Val Asp Glu Cys Ala Ser Arg Ala Ser Cys
 1060 1065 1070

Pro Thr Gly Leu Cys Leu Asn Thr Glu Gly Ser Phe Thr Cys Ser Ala
 1075 1080 1085

-242-

Cys Gln Ser Gly Tyr Trp Val Asn Glu Asp Gly Thr Ala Cys Glu Asp
 1090 1095 1100

Leu Asp Glu Cys Ala Phe Pro Gly Val Cys Pro Thr Gly Val Cys Thr
 5 1105 1110 1115 1120

Asn Thr Val Gly Ser Phe Ser Cys Lys Asp Cys Asp Gln Gly Tyr Arg
 1125 1130 1135

10 Pro Asn Pro Leu Gly Asn Arg Cys Glu Asp Val Asp Glu Cys Glu Gly
 1140 1145 1150

Pro Gln Ser Ser Cys Arg Gly Gly Glu Cys Lys Asn Thr Glu Gly Ser
 1155 1160 1165

15 Tyr Gln Cys Leu Cys His Gln Gly Phe Gln Leu Val Asn Gly Thr Met
 1170 1175 1180

Cys Glu Asp Val Asn Glu Cys Val Gly Glu Glu His Cys Ala Pro His
 20 1185 1190 1195 1200

Gly Glu Cys Leu Asn Ser Leu Gly Ser Phe Phe Cys Leu Cys Ala Pro
 1205 1210 1215

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Gly Phe Ala Ser Ala Glu Gly Gly Thr Arg Cys Gln Asp Val Asp Glu
1220 1225 1230

Cys Ala Ala Thr Asp Pro Cys Pro Gly Gly His Cys Val Asn Thr Glu
1235 1240 1245

Gly Ser Phe Ser Cys Leu Cys Glu Thr Ala Ser Phe Gln Pro Ser Pro
1250 1255 1260

10 Asp Ser Gly Glu Cys Leu Asp Ile Asp Glu Cys Glu Asp Arg Glu Asp
1265 1270 1275 1280

Pro Val Cys Gly Ala Trp Arg Cys Glu Asn Ser Pro Gly Ser Tyr Arg
1285 1290 1295

15

Cys Ile Leu Asp Cys Gln Pro Gly Phe Tyr Val Ala Pro Asn Gly Asp
1300 1305 1310

Cys Ile Asp Ile Asp Glu Cys Ala Asn Asp Thr Val Cys Gly Asn His
1315 1320 1325

20

Gly Phe Cys Asp Asn Thr Asp Gly Ser Phe Arg Cys Leu Cys Asp Gln
1330 1335 1340

-244-

Gly Phe Glu Thr Ser Pro Ser Gly Trp Glu Cys Val Asp Val Asn Glu
1345 1350 1355 1360

Cys Glu Leu Met Met Ala Val Cys Gly Asp Ala Leu Cys Glu Asn Val
1365 1370 1375

5

Glu Gly Ser Phe Leu Cys Leu Cys Ala Ser Asp Leu Glu Glu Tyr Asp
1380 1385 1390

10 Ala Glu Glu Gly His Cys Arg Pro Arg Val Ala Gly Ala Gln Arg Ile
1395 1400 1405

Pro Glu Val Arg Thr Glu Asp Gln Ala Pro Ser Leu Ile Arg Met Glu
1410 1415 1420

15

Cys Tyr Ser Glu His Asn Gly Gly Pro Pro Cys Ser Gln Ile Leu Gly
1425 1430 1435 1440

Gln Asn Ser Thr Gln Ala Glu Cys Cys Cys Thr Gln Gly Ala Arg Trp
1445 1450 1455

20

Gly Lys Ala Cys Ala Pro Cys Pro Ser Glu Asp Ser Val Glu Phe Ser
1460 1465 1470

-245-

Gln Leu Cys Pro Ser Gly Gln Gly Tyr Ile Pro Val Glu Gly Ala Trp
 1475 1480 1485

Thr Phe Gly Gln Thr Met Tyr Thr Asp Ala Asp Glu Cys Val Leu Phe
 1490 1495 1500

Gly Pro Ala Leu Cys Gln Asn Gly Arg Cys Ser Asn Ile Val Pro Gly
 1505 1510 1515 1520

10 Tyr Ile Cys Leu Cys Asn Pro Gly Tyr His Tyr Asp Ala Ser Ser Arg
 1525 1530 1535

Lys Cys Gln Asp His Asn Glu Cys Gln Asp Leu Ala Cys Glu Asn Gly
 1540 1545 1550

Glu Cys Val Asn Gln Glu Gly Ser Phe His Cys Leu Cys Asn Pro Pro
 1555 1560 1565

20 Leu Thr Leu Asp Leu Ser Gly Gln Arg Cys Val Asn Thr Thr Ser Ser
 1570 1575 1580

Thr Glu Asp Phe Pro Asp His Asp Ile His Met Asp Ile Cys Trp Lys
 1585 1590 1595 1600

-246-

Lys Val Thr Asn Asp Val Cys Ser Gln Pro Leu Arg Gly His His Thr
1605 1610 1615

Thr Tyr Thr Glu Cys Cys Gln Asp Gly Glu Ala Trp Ser Gln Gln
1620 1625 1630

5

Cys Ala Leu Cys Pro Pro Arg Ser Ser Glu Val Tyr Ala Gln Leu Cys
1635 1640 1645

Asn Val Ala Arg Ile Glu Ala Glu Arg Gly Ala Gly Ile His Phe Arg
1650 1655 1660

Pro Gly Tyr Glu Tyr Gly Pro Gly Leu Asp Asp Leu Pro Glu Asn Leu
1665 1670 1675 1680

15

Tyr Gly Pro Asp Gly Ala Pro Phe Tyr Asn Tyr Leu Gly Pro Glu Asp
1685 1690 1695

Thr Ala Pro Glu Pro Pro Phe Ser Asn Pro Ala Ser Gln Pro Gly Asp
1700 1705 1710

20

Asn Thr Pro Val Leu Glu Pro Pro Leu Gln Pro Ser Glu Leu Gln Pro
1715 1720 1725

-247-

His Tyr Leu Ala Ser His Ser Glu Pro Pro Ala Ser Phe Glu Gly Leu
 1730 1735 1740

Gln Ala Glu Glu Cys Gly Ile Leu Asn Gly Cys Glu Asn Gly Arg Cys
 5 1745 1750 1755 1760

Val Arg Val Arg Glu Gly Tyr Thr Cys Asp Cys Phe Glu Gly Phe Gln
 1765 1770 1775

10 Leu Asp Ala Pro Thr Leu Ala Cys Val Asp Val Asn Glu Cys Glu Asp
 1780 1785 1790

Leu Asn Gly Pro Ala Arg Leu Cys Ala His Gly His Cys Glu Asn Thr
 1795 1800 1805

15 Glu Gly Ser Tyr Arg Cys His Cys Ser Pro Gly Tyr Val Ala Glu Pro
 1810 1815 1820

Gly Pro Pro His Cys Ala Ala Lys Glu *
 20 1825 1830

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CLAIMS

1. A method for transferring a nucleic acid segment
5 into bone progenitor cells, comprising contacting bone progenitor cells with a composition comprising an isolated nucleic acid segment so as to transfer said nucleic acid segment into said cells.
- 10 2. The method of claim 1, wherein said cells are located within a bone progenitor tissue site of an animal and said tissue site is contacted with said composition so as to promote nucleic acid transfer into bone
15 progenitor cells *in situ*.
3. The method of claim 2, wherein the contacting
20 process comprises bringing said isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment composition and bringing said matrix-nucleic acid segment composition into contact with said tissue site.
- 25 4. The use of a composition comprising an isolated nucleic acid segment and a bone-compatible matrix in the preparation of a formulation or medicament for transferring a nucleic acid segment into bone progenitor
30 cells.
5. A use according to claim 4, wherein said formulation
35 or medicament is intended for use in transferring a nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.

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6. A use according to claim 5, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix to form a matrix-nucleic acid segment formulation or medicament intended for use in transferring a nucleic acid segment into bone progenitor cells within a bone progenitor tissue site of an animal.
7. A use according to claim 6, wherein said formulation or medicament is prepared by bringing an isolated nucleic acid segment into contact with a bone-compatible matrix and a pluronic agent to form a syringeable matrix-nucleic acid segment formulation or medicament.
8. A use according to claim 6, wherein said formulation or medicament further comprises a detectable agent for use in an imaging modality.
9. A use according to claim 8, wherein said formulation or medicament further comprises a radiographic agent.
10. A use according to claim 8, wherein said formulation or medicament further comprises a paramagnetic ion.
11. A use according to claim 8, wherein said formulation or medicament further comprises a radioactive ion.
12. A use according to claim 4, wherein said nucleic acid segment is a DNA molecule.

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13. A use according to claim 4, wherein said nucleic acid segment is an RNA molecule.

5 14. A use according to claim 4, wherein said nucleic acid segment is an antisense nucleic acid molecule.

10 15. A use according to claim 4, wherein said nucleic acid segment is a linear nucleic acid molecule, a plasmid, a recombinant insert within the genome of a recombinant virus, or a nucleic acid segment associated with a liposome.

15 16. A use according to claim 15, wherein said nucleic acid segment is a nucleic acid segment associated with a liposome.

20 17. A use according to claim 4, wherein said nucleic acid segment encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells.

25 18. A use according to claim 6, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

30

19. A use according to claim 18, wherein said bone-compatible matrix is a titanium matrix.

35

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20. A use according to claim 19, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

5

21. A use according to claim 18, wherein said bone-compatible matrix is a collagen preparation.

10

22. A use according to claim 21, wherein said bone-compatible matrix is a type II collagen preparation.

15

23. A use according to claim 22, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

20

24. A use according to claim 22, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

25

25. A use according to claim 22, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

30

26. A method of stimulating bone progenitor cells, comprising contacting bone progenitor cells with a composition comprising an isolated osteotropic gene so as to promote expression of said gene in said cells.

35

27. The method of claim 26, wherein said cells are located within a bone progenitor tissue site of an animal

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and said tissue site is contacted with said composition so as to promote bone tissue growth.

5 28. The method of claim 27, wherein the contacting process comprises bringing said osteotropic gene into contact with a bone-compatible matrix to form a matrix-gene composition and bringing said matrix-gene composition into contact with said tissue site.

10

29. The use of a composition comprising an isolated osteotropic gene in the preparation of a formulation or medicament for use in promoting expression of the gene in
15 bone progenitor cells and for stimulating said bone progenitor cells.

30. A use according to claim 29, wherein said
20 formulation or medicament is intended for use in promoting expression of the gene in bone progenitor cells within a bone progenitor tissue site of an animal and for stimulating said bone progenitor cells to promote bone tissue growth.

25

31. A use according to claim 30, wherein said formulation or medicament is prepared by bringing an osteotropic gene into contact with a bone-compatible
30 matrix to form a matrix-gene formulation or medicament intended for use in promoting expression of the gene in bone progenitor cells within a bone progenitor tissue site of an animal and for stimulating said bone progenitor cells to promote bone tissue growth.

35

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32. A use according to claim 31, wherein said
formulation or medicament is prepared by bringing an
osteotropic gene into contact with a bone-compatible
matrix and a pluronic agent to form a syringeable matrix-
5 gene formulation or medicament.

33. A use according to claim 31, wherein said
formulation or medicament further comprises a detectable
10 agent for use in an imaging modality.

34. A use according to claim 33, wherein said
formulation or medicament further comprises a
15 radiographic agent.

35. A use according to claim 34, wherein said
formulation or medicament further comprises calcium
20 phosphate.

36. A use according to claim 33, wherein said
formulation or medicament further comprises a
paramagnetic ion.
25

37. A use according to claim 36, wherein said
formulation or medicament further comprises chromium
(III), manganese (II), iron (III), iron (II), cobalt
30 (II), nickel (II), copper (II), neodymium (III), samarium
(III), ytterbium (III), gadolinium (III), vanadium (II),
terbium (III), dysprosium (III), holmium (III) or
erbium (III).

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38. A use according to claim 33, wherein said formulation or medicament further comprises a radioactive ion.

5

39. A use according to claim 38, wherein said formulation or medicament further comprises iodine¹³¹, iodine¹²³, technetium^{99m}, indium¹¹¹, rhenium¹⁸⁸, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰, iodine¹²⁵ or astatine²¹¹.

10

40. A use according to claim 29, wherein said osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adeno-associated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated with a liposome.

20

41. A use according to claim 40, wherein said osteotropic gene is in the form of an osteotropic gene associated with a liposome.

25

42. A use according to claim 29, wherein said osteotropic gene is a parathyroid hormone (PTH) gene, a bone morphogenetic protein (BMP) gene, a growth factor gene, a growth factor receptor gene, a cytokine gene or a chemotactic factor gene.

30

43. A use according to claim 42, wherein said osteotropic gene is a transforming growth factor (TGF) gene, a fibroblast growth factor (FGF) gene, a granulocyte/macrophage colony stimulating factor (GMCSF)

35

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gene, an epidermal growth factor (EGF) gene, a platelet derived growth factor (PDGF) gene, an insulin-like growth factor (IGF) gene, or a leukemia inhibitory factor (LIF) gene.

5

44. A use according to claim 43, wherein said osteotropic gene is a TGF- α , TGF- β 1 or TGF- β 2 gene.

10

45. A use according to claim 42, wherein said osteotropic gene is a PTH gene.

15

46. A use according to claim 42, wherein said osteotropic gene is a BMP gene.

20

47. A use according to claim 46, wherein said osteotropic gene is a BMP-2 or BMP-4 gene.

25

48. A use according to claim 31, wherein said bone-compatible matrix is a collagenous, metal, hydroxylapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

30

49. A use according to claim 48, wherein said bone-compatible matrix is a titanium matrix.

35

50. A use according to claim 49, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

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51. A use according to claim 48, wherein said bone-compatible matrix is a collagen preparation.

5 52. A use according to claim 51, wherein said bone-compatible matrix is a type II collagen preparation.

10 53. A use according to claim 52, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

15 54. A use according to claim 52, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

20 55. A use according to claim 52, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

25 56. A use according to claim 31, wherein said matrix-gene composition is applied to a bone fracture site in said animal.

30 57. A use according to claim 31, wherein said matrix-gene composition is implanted within a bone cavity site in said animal.

35 58. A use according to claim 31, wherein said bone cavity site is the result of dental or periodontal surgery or the removal of an osteosarcoma.

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59. A composition comprising an isolated nucleic acid segment in association with a bone-compatible matrix.

5 60. The composition of claim 59, wherein said nucleic acid segment is a DNA molecule.

10 61. The composition of claim 59, wherein said nucleic acid segment is an RNA molecule.

15 62. The composition of claim 59, wherein said nucleic acid segment is an antisense nucleic acid molecule.

63. The composition of claim 59, wherein said nucleic acid segment is a linear nucleic acid molecule, a plasmid, a recombinant insert within the genome of a recombinant virus, or a nucleic acid segment associated with a liposome.

25 64. The composition of claim 63, wherein said nucleic acid segment is associated with a liposome.

65. The composition of claim 59, wherein said nucleic acid segment encodes a polypeptide or protein that stimulates bone progenitor cells when expressed in said cells.

30 66. The composition of claim 59, wherein said bone-compatible matrix is a collagenous, titanium, hydroxylapatite, hydroxylapatite-coated titanium, bioglass, aluminate, bioceramic, acrylic ester polymer or

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lactic acid polymer matrix.

5 67. The composition of claim 66, wherein said bone-compatible matrix is a collagen preparation.

10 68. The composition of claim 67, wherein said bone-compatible matrix is a type II collagen preparation.

15 69. The composition of claim 68, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

20 70. The composition of claim 68, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

25 71. The composition of claim 68, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

72. The composition of claim 59, further defined as a syringeable composition.

30 73. The composition of claim 59, wherein said composition further comprises a detectable agent for use in an imaging modality.

35

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74. The composition of claim 73, wherein said composition further comprises a radiographic agent.

5 75. The composition of claim 73, wherein said composition further comprises a paramagnetic ion.

10 76. The composition of claim 73, wherein said composition further comprises a radioactive ion.

15 77. A composition comprising an isolated osteotropic gene in association with a bone-compatible matrix; said composition being capable of stimulating bone growth when administered to a bone progenitor tissue site of an animal.

20 78. The composition of claim 77, wherein said osteotropic gene is in the form of plasmid DNA, a DNA insert within the genome of a recombinant adenovirus, a DNA insert within the genome of a recombinant adeno-associated virus (AAV), a DNA insert within the genome of a recombinant retrovirus, or a DNA segment associated
25 with a liposome.

30 79. The composition of claim 78, wherein said osteotropic gene is in the form of an osteotropic gene associated with a liposome.

35 80. The composition of claim 77, wherein said osteotropic gene is a PTH, BMP, TGF- α , TGF- β 1, TGF- β 2, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.

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81. The composition claim 80, wherein said osteotropic gene is a TGF- α , TGF- β 1, TGF- β 2, PTH, BMP-2 or BMP-4 gene.

5

82. The composition of claim 77, wherein said bone-compatible matrix is a collagenous, metal, hydroxyapatite, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer matrix.

10

83. The composition of claim 82, wherein said bone-compatible matrix is a titanium matrix.

15

84. The composition of claim 83, wherein said bone-compatible matrix is a titanium matrix coated with hydroxylapatite.

20

85. The composition of claim 82, wherein said bone-compatible matrix is a collagen preparation.

25

86. The composition of claim 85, wherein said bone-compatible matrix is a type II collagen preparation.

30

87. The composition of claim 86, wherein said bone-compatible matrix is a type II collagen preparation obtained from hyaline cartilage.

35

88. The composition of claim 86, wherein said bone-compatible matrix is a recombinant type II collagen preparation.

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89. The composition of claim 86, wherein said bone-compatible matrix is a mineralized type II collagen preparation.

5

90. The composition of claim 77, further defined as comprising an isolated osteotropic gene in association with a bone-compatible matrix and a pluronic agent, the composition forming a syringeable composition.

10

91. The composition of claim 77, wherein said composition further comprises a detectable agent for use in an imaging modality.

15

92. The composition of claim 91, wherein said composition further comprises a radiographic agent.

20

93. The composition of claim 92, wherein said composition further comprises calcium phosphate.

25

94. The composition of claim 91, wherein said composition further comprises a paramagnetic ion.

30

95. The composition of claim 94, wherein said composition further comprises chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

35

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96. The composition of claim 91, wherein said composition further comprises a radioactive ion.

5 97. The composition of claim 96, wherein said composition further comprises iodine¹³¹, iodine¹²³, technetium^{99m}, indium¹¹¹, rhenium¹⁸⁸, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰, iodine¹²⁵ or astatine²¹¹.

10

98. A kit comprising, in suitable container means, a pharmaceutically acceptable bone-compatible matrix and a pharmaceutically acceptable osteotropic gene preparation.

15

99. The kit of claim 98, wherein said bone-compatible matrix is a collagenous, titanium, hydroxylapatite, hydroxylapatite-coated titanium, bioglass, aluminate, bioceramic, acrylic ester polymer or lactic acid polymer
20 matrix.

100. The kit of claim 99, wherein said bone-compatible matrix is a titanium matrix.

25

101. The kit of claim 99, wherein said bone-compatible matrix is a hydroxylapatite-coated titanium matrix.

30 102. The kit of claim 99, wherein said bone-compatible matrix is a collagenous matrix.

103. The kit of claim 102, wherein said bone-compatible
35 matrix is a type II collagen matrix.

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104. The kit of claim 103, wherein said bone-compatible matrix is a type II collagen matrix obtained from hyaline cartilage.

5

105. The kit of claim 103, wherein said bone-compatible matrix is a recombinant type II collagen matrix.

10

106. The kit of claim 103, wherein said bone-compatible matrix is a mineralized type II collagen matrix.

15

107. The kit of claim 98, wherein said osteotropic gene preparation comprises a linear osteotropic gene, a plasmid including an osteotropic gene, a recombinant virus having a genome that includes an osteotropic gene or an osteotropic gene associated with a liposome.

20

108. The kit of claim 98, wherein said osteotropic gene preparation comprises a lyophilized gene preparation.

25

109. The kit of claim 98, wherein said osteotropic gene preparation comprises a PTH, TGF, BMP, FGF, GMCSF, EGF, PDGF, IGF or a LIF gene.

30

110. The kit of claim 109, wherein said osteotropic gene preparation comprises a PTH, TGF- β 1, TGF- β 2, TGF- β 3, BMP-2 or a BMP-4 gene.

35

111. The kit of claim 98, further comprising a pluronic agent.

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112. The kit of claim 98, further comprising a detectable agent for use in an imaging modality.

5

113. The kit of claim 112, wherein said composition further comprises a radiographic agent.

10

114. The kit of claim 113, wherein said composition further comprises calcium phosphate.

15

115. The kit of claim 112, wherein said composition further comprises a paramagnetic ion.

20

116. The kit of claim 115, wherein said composition further comprises chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) or erbium (III).

25

117. The kit of claim 112, wherein said composition further comprises a radioactive ion.

30

118. The kit of claim 117, wherein said composition further comprises iodine¹³¹, iodine¹²³, technetium^{99m}, indium¹¹¹, rhenium¹⁸⁸, rhenium¹⁸⁶, gallium⁶⁷, copper⁶⁷, yttrium⁹⁰, iodine¹²⁵ or astatine²¹¹.

35

119. The kit of claim 98, wherein said bone-compatible

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matrix and said osteotropic gene preparation are present within a single container means.

5 120. The kit of claim 119, wherein said container means is a syringe or pipette.

10 121. The kit of claim 98, wherein said bone-compatible matrix and said osteotropic gene preparation are present within distinct container means.

15 122. The kit of claim 98, further comprising a third container means comprising a pharmaceutically acceptable diluent.

20 123. The kit of claim 98, further comprising a syringe, pipette or forceps.

25 124. An osteotropic device, comprising an isolated osteotropic gene capable of expression in bone progenitor cells, the gene associated with an amount of a bone-compatible matrix effective to absorb said gene, wherein said device is capable of stimulating bone formation when implanted within a bone progenitor tissue site of an animal.

30 125. The device of claim 124, wherein said device is a titanium or a hydroxylapatite-coated titanium device.

35 126. The device of claim 124, wherein said device is shaped to join a bone fracture site in said animal.

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127. The device of claim 124, wherein said device is shaped to fill a bone cavity site in said animal.

5

128. The device of claim 124, wherein said device is an artificial joint.

10

129. A DNA segment comprising an isolated gene that encodes a protein or peptide that includes an amino acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:3.

15

130. The DNA segment of claim 129, comprising an isolated gene that includes a nucleic acid sequence essentially as set forth by a contiguous sequence from SEQ ID NO:2.

20

131. A composition comprising a purified protein that has an amino acid sequence essentially as set forth by the amino acid sequence of SEQ ID NO:3.

25

132. A method for stimulating a bone progenitor cell, comprising contacting a bone progenitor cell with a composition comprising a biologically effective amount of type II collagen.

30

133. The use of a composition comprising a biologically effective amount of type II collagen in the preparation of a formulation or medicament for stimulating a bone progenitor cell.

35

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134. A use according to claim 133, wherein said composition comprises type II collagen purified from hyaline cartilage.

5

135. A use according to claim 133, wherein said composition comprises recombinant type II collagen.

10 136. A use according to claim 133, wherein said composition comprises type II collagen further supplemented with minerals.

15 137. A use according to claim 136, wherein said composition comprises type II collagen further supplemented with calcium.

20 138. A use according to claim 133, wherein said composition comprises between about 1 mg and about 500 mg of type II collagen.

25 139. A use according to claim 138, wherein said composition comprises between about 1 mg and about 100 mg of type II collagen.

30 140. A use according to claim 139, wherein said composition comprises about 10 mg of type II collagen.

35 141. A use according to claim 133, wherein said composition comprises type II collagen in combination with a nucleic acid segment that encodes a polypeptide or

- 268 -

protein that stimulates bone progenitor cells when expressed in said cells.

- 5 142. A use according to claim 141, wherein said nucleic acid segment comprises an isolated PTH gene, BMP gene, growth factor gene, growth factor receptor gene, cytokine gene or chemotactic factor gene.
- 10 143. A use according to claim 142, wherein said nucleic acid segment comprises an isolated BMP gene.
- 15 144. A use according to claim 143, wherein said nucleic acid segment comprises an isolated BMP-2 or BMP-4 gene.
- 20 145. A use according to claim 141, wherein said composition further comprises a detectable agent for use in an imaging modality.
- 25 146. A use according to claim 133, wherein said formulation or medicament is intended for use in stimulating a bone progenitor cell located within a bone progenitor tissue site of an animal and for promoting bone tissue growth.
- 30 147. A use according to claim 146, wherein said formulation or medicament is intended for use in implantation within a bone cavity site in an animal and for promoting bone tissue growth in said bone cavity
- 35 site.

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148. A use according to claim 146, wherein said formulation or medicament is intended for use in implantation within a bone fracture site in an animal and for promoting bone tissue growth in said bone fracture site.

149. A method for promoting bone growth, comprising contacting a bone progenitor tissue site of an animal with a composition comprising type II collagen in an amount effective to activate bone progenitor cells of said tissue site.

150. The use of a composition comprising a biologically effective amount of type II collagen in the preparation of a formulation or medicament for promoting bone growth in a bone progenitor tissue site of an animal.

151. A use according to claim 150, wherein said composition comprises recombinant type II collagen.

152. A use according to claim 150, wherein said composition comprises type II collagen further supplemented with minerals.

153. A use according to claim 150, wherein said composition comprises type II collagen and an osteotropic gene in a combined amount effective to activate bone progenitor cells of said tissue site.

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154. A use according to claim 153, wherein said composition comprises type II collagen in combination with a PTH, TGF- β or BMP gene.

5

155. A use according to claim 153, wherein said composition further comprises a detectable agent for use in an imaging modality.

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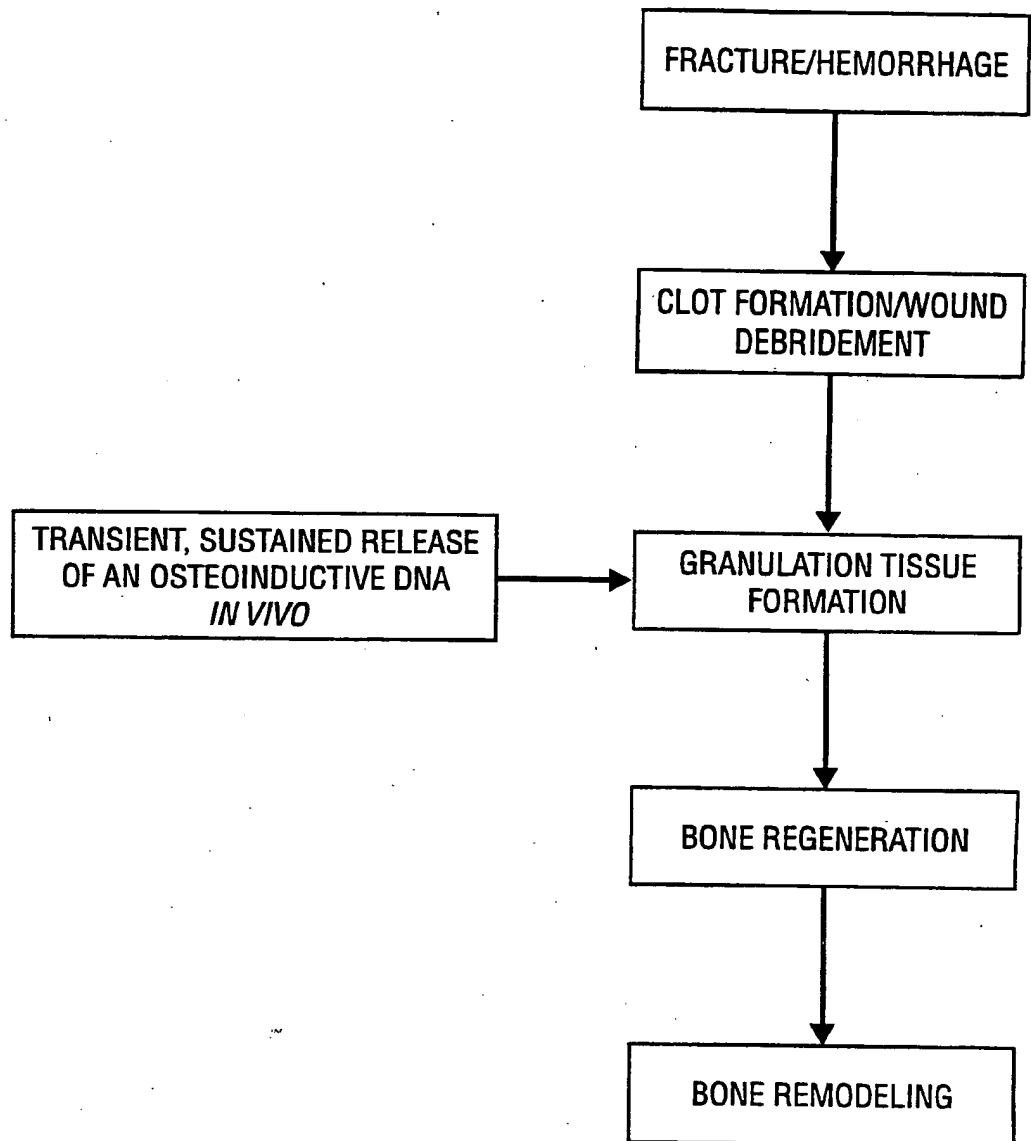


FIG. 1

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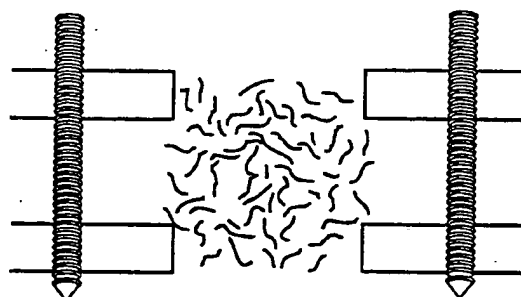


FIG. 2A

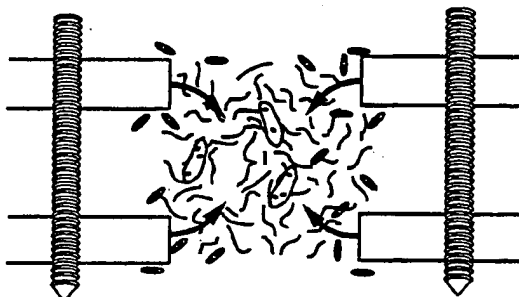


FIG. 2B

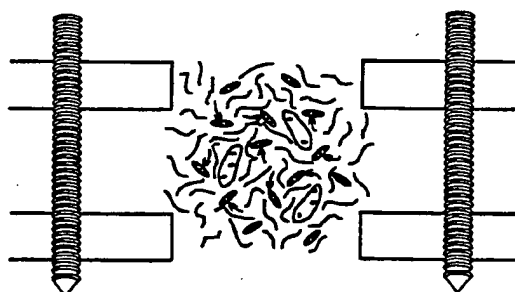


FIG. 2C

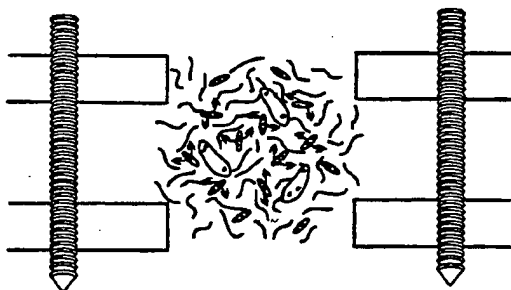


FIG. 2D

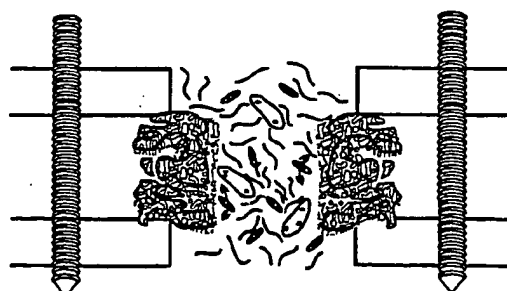


FIG. 2E

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FIG. 3A

FIG. 3B

FIG. 3C

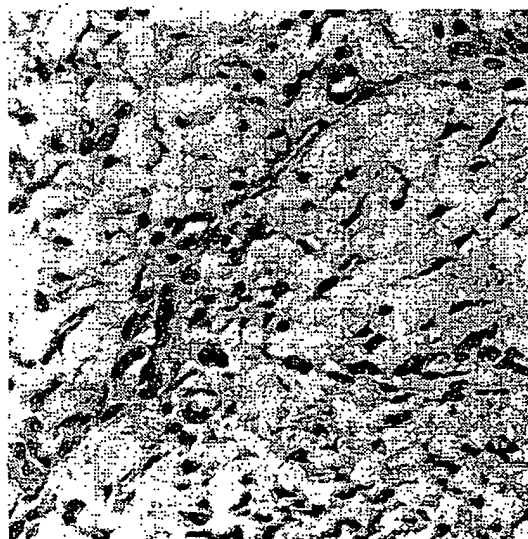


FIG. 3D

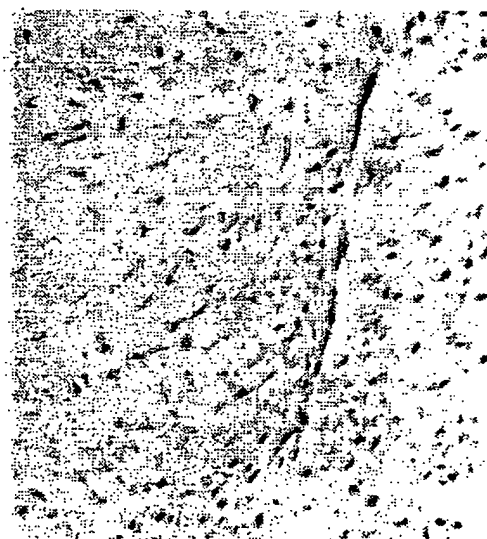


FIG. 3E

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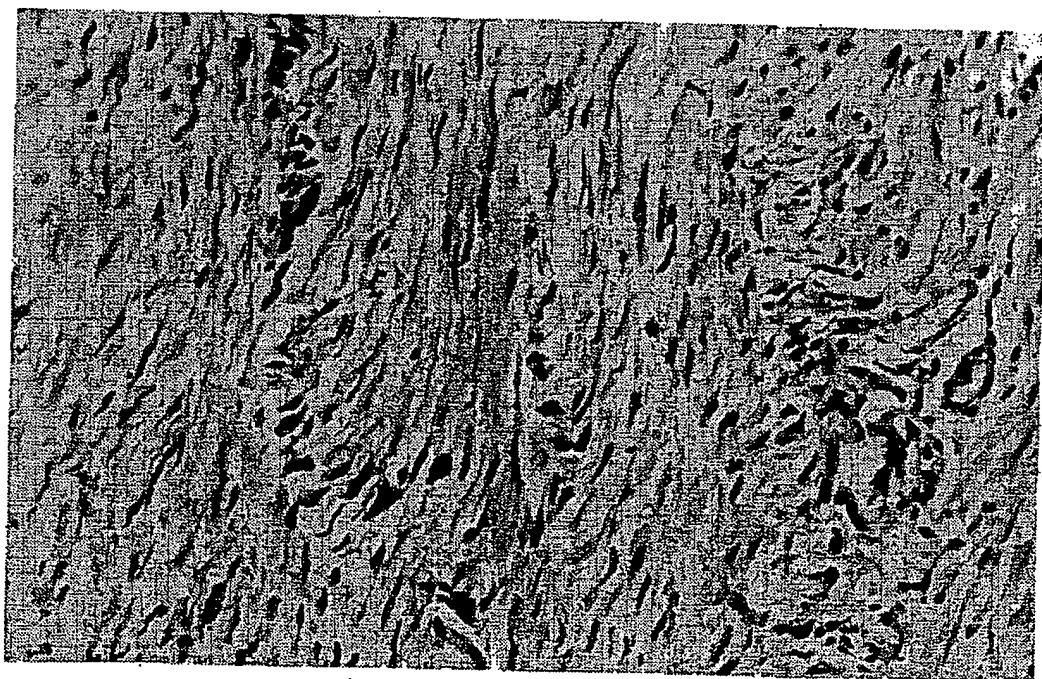


FIG. 4

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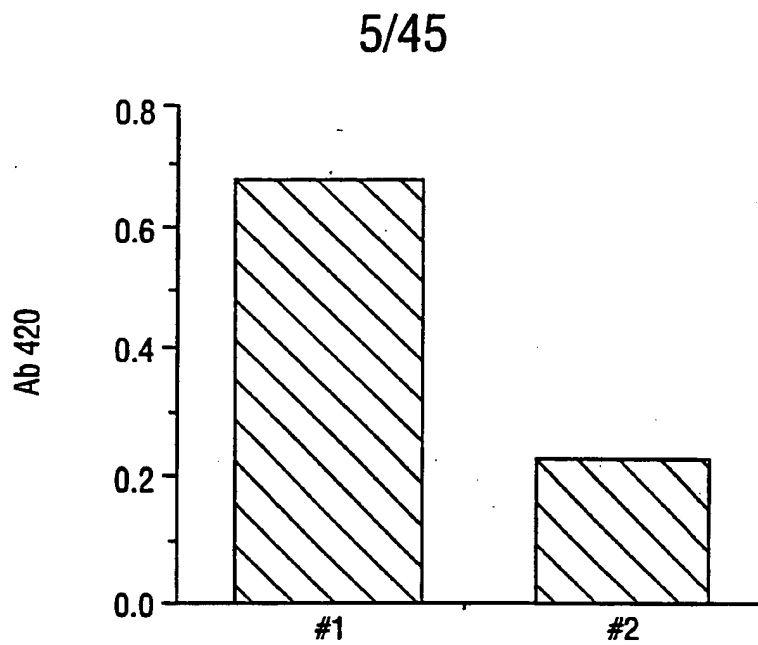


FIG. 5A

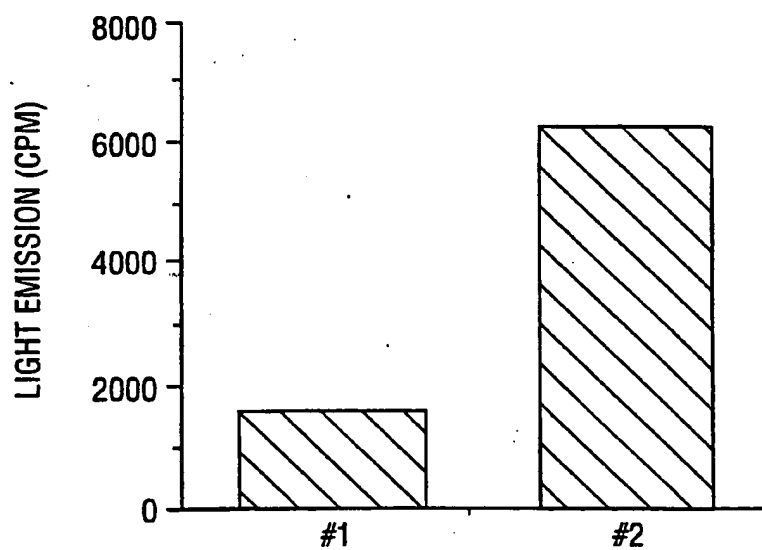


FIG. 5B



FIG. 6A



FIG. 6B



FIG. 6C

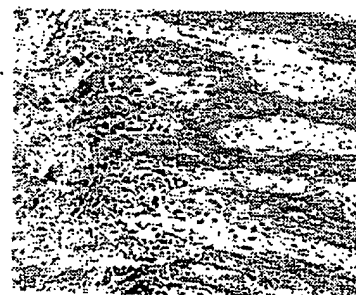


FIG. 6D

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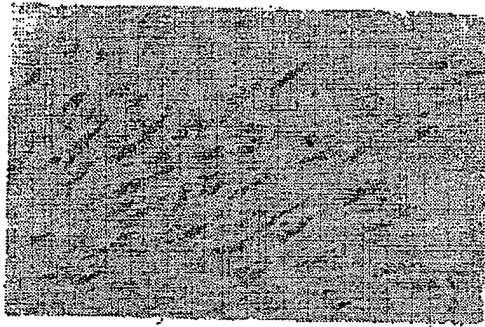


FIG. 7A



FIG. 7B

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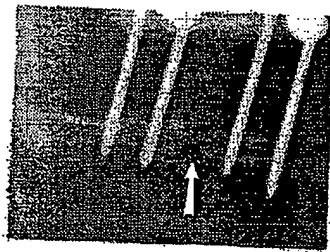


FIG. 8A

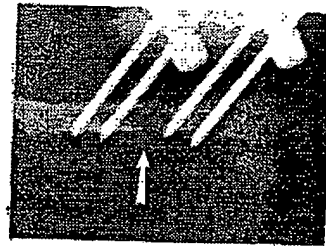


FIG. 8B



FIG. 8C

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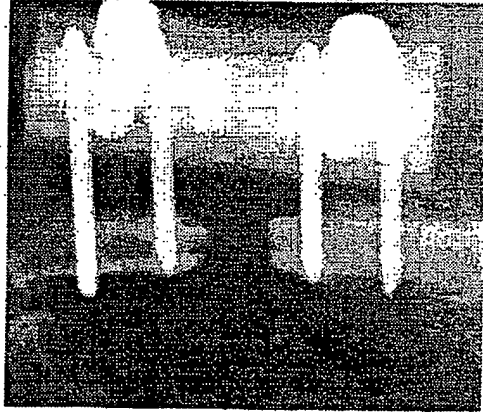


FIG. 9A

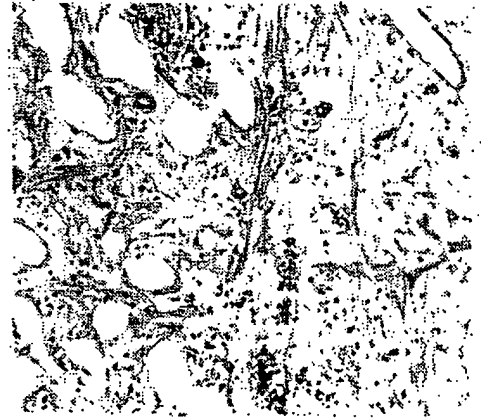
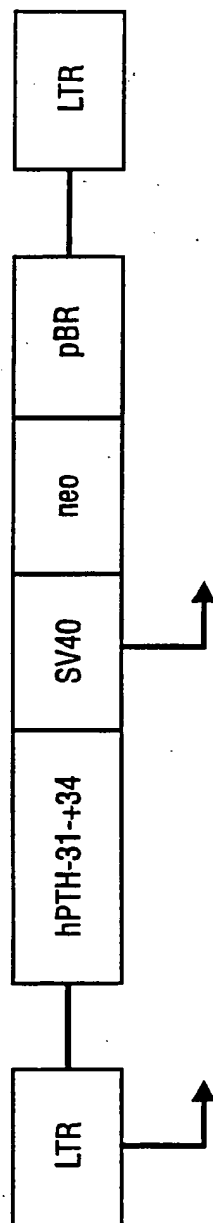


FIG. 9B

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PLJ-hPTH 1-34

FIG. 10



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1 2 3 4



4.3—

FIG. 11

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4.4 -   hPTH1-34

7.5 -   β -gal

4.4 -   Neo

2.4 -    β -actin

FIG. 12

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CONTROL
FEMUR

OSTEOTOMY
FEMUR

FIG. 13

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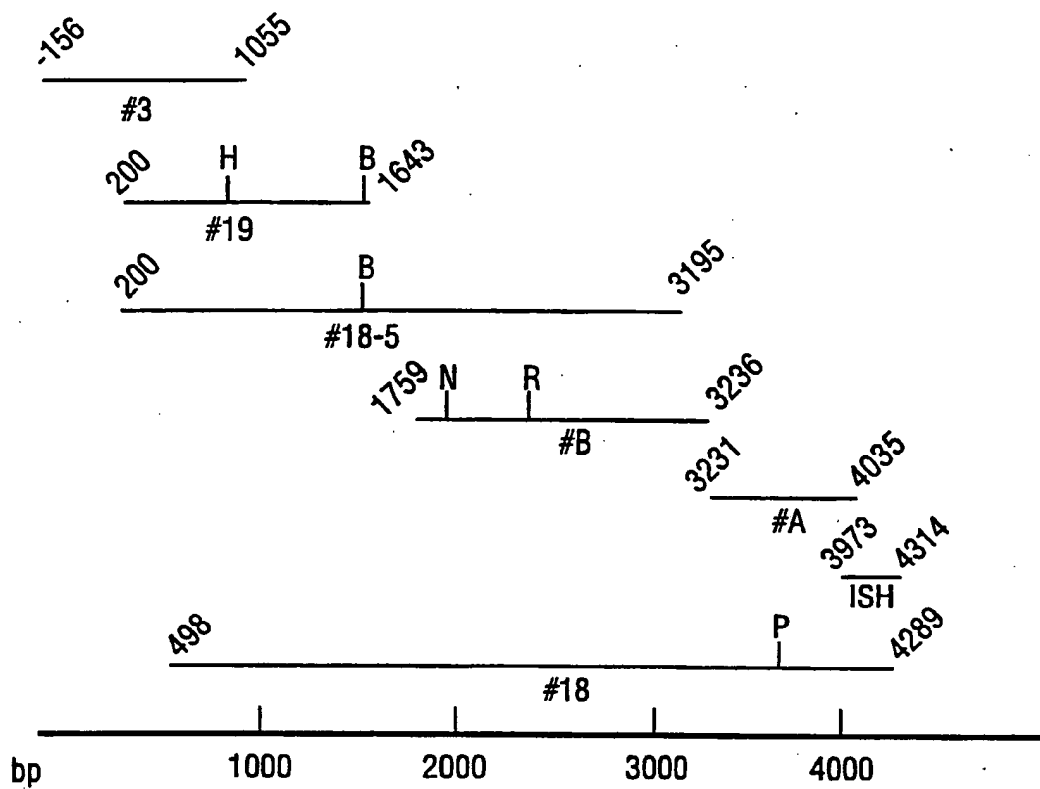


FIG. 14

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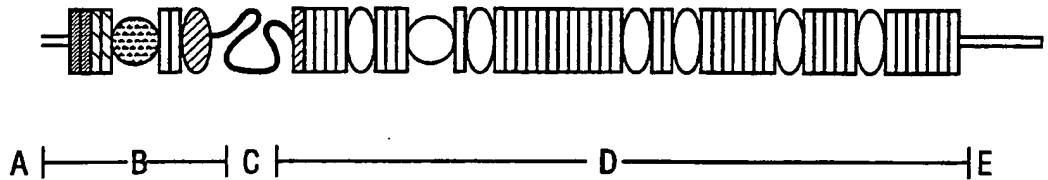


FIG. 15A

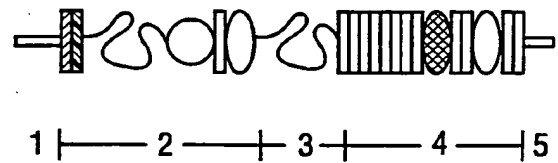


FIG. 15B

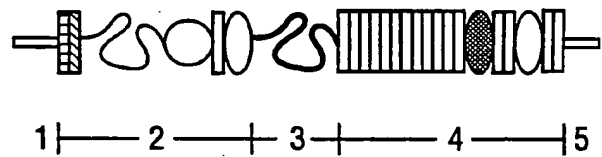


FIG. 15C

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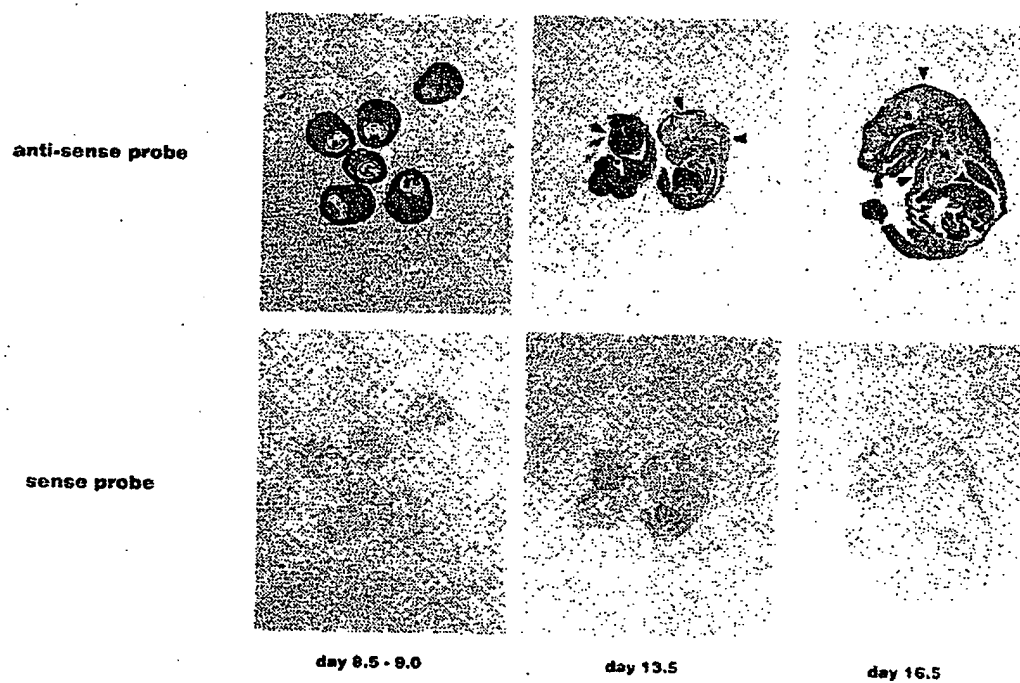


FIG. 16

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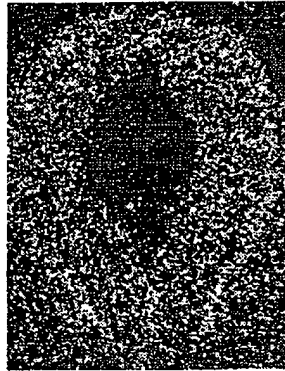


FIG. 17A

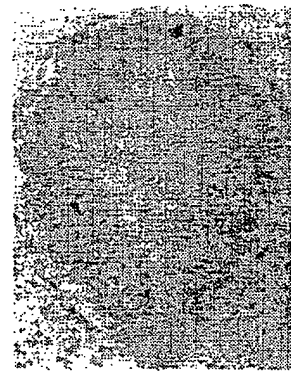


FIG. 17B

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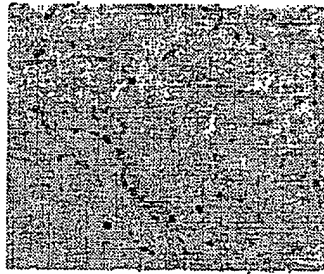


FIG. 17C

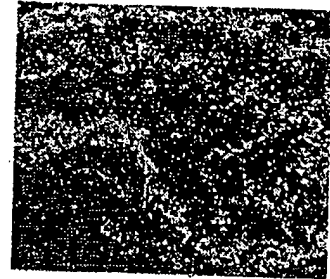


FIG. 17D

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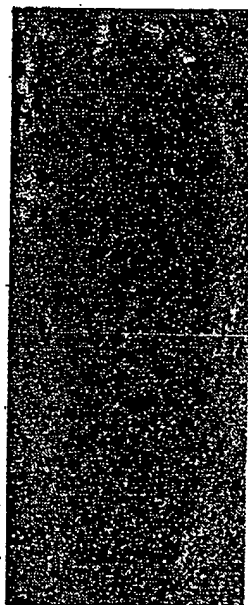


FIG. 18A

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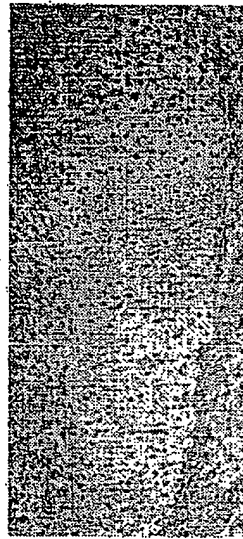


FIG. 18B

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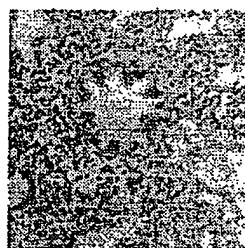


FIG. 18C

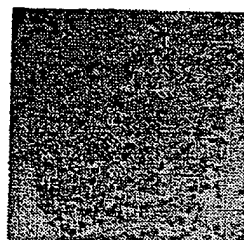


FIG. 18D

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FIG. 18E



FIG. 18F

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FIG. 18G



FIG. 18H

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FIG. 18I



FIG. 18J

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FIG. 18K



FIG. 18L

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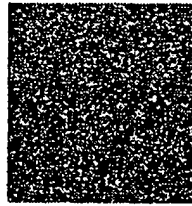


FIG. 18M



FIG. 18N

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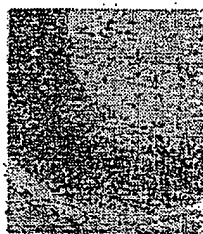


FIG. 180

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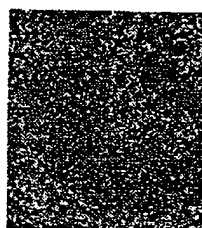
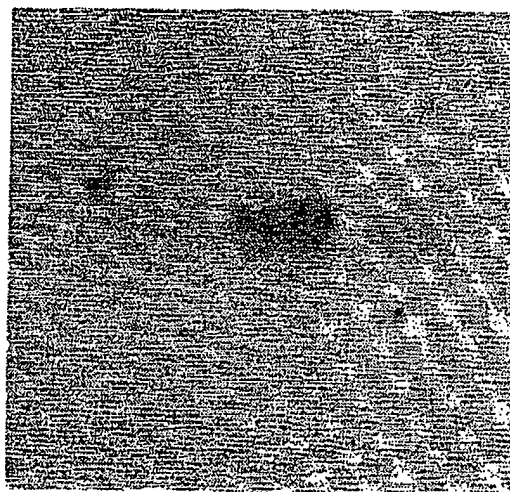


FIG. 18P

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4.4 kb—



DAY

5

14

28

FIG. 19

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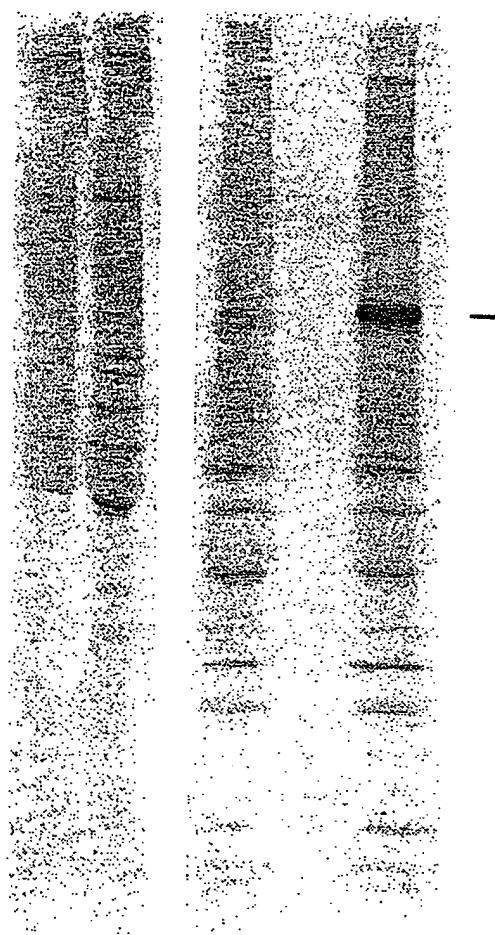


FIG. 20

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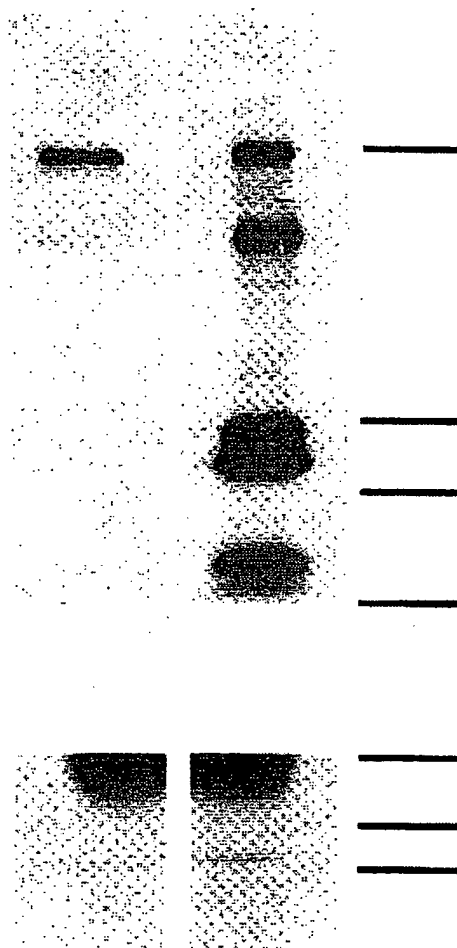


FIG. 21

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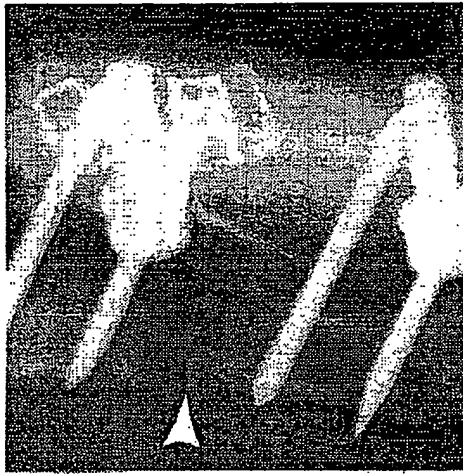


FIG. 22A

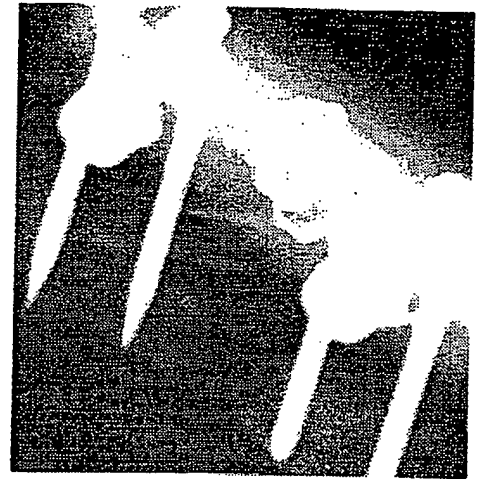


FIG. 22B



FIG. 22C

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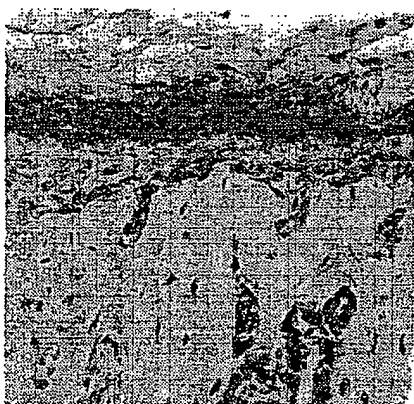


FIG. 23A

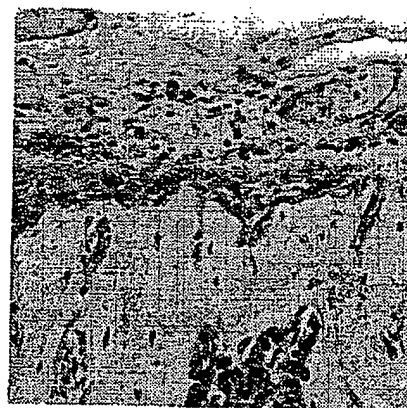


FIG. 23B

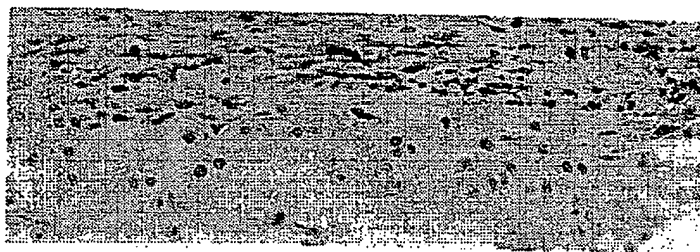


FIG. 23C

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MIPGNRMLMV VLLCQVLLGG ATDASLMPET GKKKVAEIQG HAGRRSGQS HELLRDPEAT LLQMFGLRRR
POPSKSAVIP DYMSDLYRLQ SGESEEEEQS QGTGLEYPER PASSANTVSS FHHEHLENI PGTSESSAFR
FFFNLSIPE NEVISSAELR LFREQVDQGP DWEQGFHRMN IYEVNKPPE MYPGHLITRL LDTSIVRHNV
TRWETFVSP AVLRTREKQ PNYGLAIEVT HLHQTNRTHQG QHVSISRSLP QSGNWAQLR PLLVTFGHDG
RGHTLTRRSA KRSPKHHQPOR SSKKNKNCRR HSLYVDFSDV GWNDWIVAPP GYQAFYCHGD CFPPLADHLN
STNHAIVQTL VNSVNSSIPK ACCVPTLSA ISNLYLDEYD KVVLNKYQEM VVEGCGCRYP YDVPDYA

FIG. 24

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ATG CGC CAG GCC GCA TTG GGG CTG CTG GCA CTA CTC CTG CTG GCG CTG CTG GGC 54
 M R Q A A L G L L A L L L L A L L G 18
 CCC GGC GGC CGA GGG GTG GGC CGG CCG GGC AGC GGG GCA CAG GCG GGG GCG GGG 108
 P G G R G V G R P G S G A Q A G A G 36
 CGC TGG GCC CAA CGC TTC AAG GTG GTC TTT GCG CCT GTG ATC TGC AAG CGG ACC 162
 R W A Q R F K V V F A P V I C K R T 54
 TGT CTG AAG GGC CAG TGT CGG GAC AGC TGT CAG CAG GGC TCC AAC ATG ACG CTC 216
 C L K G Q C R D S C Q Q G S N M T L 72
 ATC GGA GAG AAC GGC CAC AGC ACC GAC ACG CTC ACC GGT TCT GCC TTC CGC GTG 270
 I G E N G H S T D T L T G S A F R V 90
 GTG GTG TGC CCT CTA CCC TGC ATG AAC GGT GGC CAG TGC TCT TCC CGA AAC CAG 324
 V V C P L P C M N G G Q C S S R N Q 108
 TGC CTG TGT CCC CCG GAT TTC ACG GGG CGC TTC TGC CAG GTG CCT GCT GCA GGA 378
 C L C P P D F T G R F C Q V P A A G 126
 ACC GGA GCT GGC ACC GGG AGT TCA GGC CCC GGC TGG CCC GAC CGG GCC ATG TCC 432
 T G A G T G S S G P G W P D R A M S 144
 ACA GGC CCG CTG CCG CCC CTT GCC CCA GAA GGA GAG TCT GTG GCT AGC AAA CAC 486
 T G P L P P L A P E G E S V A S K H 162
 GCC ATT TAC GCG GTG CAG GTG ATC GCA GAT CCT CCC GGG CCG GGG GAG GGT CCT 540
 A I Y A V Q V I A D P P G P G E G P 180
 CCT GCA CAA CAT GCA GCC TTC TTG GTG CCC CTG GGG CCA GGA CAA ATC TCG GCA 594
 P A Q H A A F L V P L G P G Q I S A 198
 GAA GTG CAG GCT CCG CCC CCC GTG GTG AAC GTG CGT GTC CAT CAC CCT CCT GAA 648
 E V Q A P P P V V N V R V H H P P E 216
 GCT TCC GTT CAG GTG CAC CGC ATC GAG GGG CCG AAC GCT GAA GGC CCA GCC TCT 702
 A S V Q V H R I E G P N A E G P A S 234
 TCC CAG CAC TTG CTG CCG CAT CCC AAG CCC CAG CAC CCG AGG CCA CCC ACT CAA 756
 S Q H L L P H P K P Q H P R P P T Q 252
 AAG CCA CTG GGC CGC TGC TTC CAG GAC ACA TTG CCC AAG CAG CCT TGT GGC AGC 810
 K P L G R C F Q D T L P K Q P C G S 270
 AAC CCT TTG CCT GGC CTT ACC AAG CAG GAA GAT TGC TGC GGT AGC ATC GGT ACT 864
 N P L P G L T K Q E D C C G S I G T 288
 GCC TGG GGA CAA AGC AAG TGT CAC AAG TGC CCA CAG CTT CAG TAT ACA GGG GTG 918
 A W G Q S K C H K C P Q L Q Y T G V 306
 CAG AAG CCT GTA CCT GTA CGT GGG GAG GTG GGT GCT GAC TGC CCC CAG GGC TAC 972
 Q K P V P V R G E V G A D C P Q G Y 324
 AAG AGG CTC AAC AGC ACC CAC TGC CAG GAT ATC AAC GAA TGT GCG ATG CCC GGG 1026
 K R L N S T H C Q D I N E C A M P G 342

FIG. 25-1

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AAT	GTG	TGC	CAT	GGT	GAC	TGC	CTC	AAC	AAC	CCT	GGC	TCT	TAT	CGC	TGT	GTC	TGC	1080
N	V	C	H	G	D	C	L	N	N	P	G	S	Y	R	C	V	C	360
CCG	CCC	GGT	CAT	AGC	TTG	GGT	CCC	CTC	GCA	GCA	CAG	TGC	ATT	GCC	GAC	AAA	CCA	1134
P	P	G	H	S	L	G	P	L	A	A	Q	C	I	A	D	K	P	378
GAG	GAG	AAG	AGC	CTG	TGT	TTC	CGC	CTT	GTG	AGC	ACC	GAA	CAC	CAG	TGC	CAG	CAC	1188
E	E	K	S	L	C	F	R	L	V	S	T	E	H	Q	C	Q	H	396
CCT	CTG	ACC	ACA	CGC	CTA	ACC	CGC	CAG	CTC	TGC	TGC	TGT	AGT	GTG	GGT	AAA	GCC	1242
P	L	T	T	R	L	T	R	Q	L	C	C	C	S	V	G	K	A	414
TGG	GGT	GCC	CGG	TGC	CAG	CGC	TGC	CCG	GCA	GAT	GGT	ACA	GCA	GCC	TTC	AAG	GAG	1296
W	G	A	R	C	Q	R	C	P	A	D	G	T	A	A	F	K	E	432
ATC	TGC	CCC	GGC	TGG	GAA	AGG	GTA	CCA	TAT	CCT	CAC	CTC	CCA	CCA	GAC	GCT	CAC	1350
I	C	P	G	W	E	R	V	P	Y	P	H	L	P	P	D	A	H	450
CAT	CCA	GGG	GGA	AAG	CGA	CTT	CTC	CCT	CTT	CCT	GCA	CCC	GAC	GGG	CCA	CCC	AAA	1404
H	P	G	G	K	R	L	L	P	L	P	A	P	D	G	P	P	K	468
CCC	CAG	CAG	CTT	CCT	GAA	AGC	CCC	AGC	CGA	GCA	CCA	CCC	CTC	GAG	GAC	ACA	GAG	1458
P	Q	Q	L	P	E	S	P	S	R	A	P	P	L	E	D	T	E	486
GAA	GAG	AGA	GGA	GTG	ACC	ATG	GAT	CCA	CCA	GTG	AGT	GAG	GAG	CGA	TCG	GTG	CAG	1512
E	E	R	G	V	T	M	D	P	P	V	S	E	E	R	S	V	Q	504
CAG	AGC	CAC	CCC	ACT	ACC	ACC	ACC	TCA	CCC	CCC	CGG	CCT	TAC	CCA	GAG	CTC	ATC	1566
Q	S	H	P	T	T	T	T	S	P	P	R	P	Y	P	E	L	I	522
TCT	CGC	CCC	TCC	CCA	CCT	ACC	TTC	CAC	CGG	TTC	CTG	CCA	GAC	TTG	CCC	CCA	TCC	1620
S	R	P	S	P	P	T	F	H	R	F	L	P	D	L	P	P	S	540
CGA	AGT	GCA	GTG	GAG	ATC	GCC	CCC	ACT	CAG	GTC	ACA	GAG	ACC	GAT	GAG	TGC	CGA	1674
R	S	A	V	E	I	A	P	T	Q	V	T	E	T	D	E	C	R	558
TTG	AAC	CAG	AAT	ATC	TGT	GGC	CAT	GGA	CAG	TGT	GTG	CCT	GGC	CCC	TCG	GAT	TAC	1728
L	N	Q	N	I	C	G	H	G	Q	C	V	P	G	P	S	D	Y	576
TCC	TGC	CAC	TGC	AAC	GCT	GGC	TAC	CGG	TCA	CAC	CCG	CAG	CAC	CGC	TAC	TGT	GTT	1782
S	C	H	C	N	A	G	Y	R	S	H	P	Q	H	R	Y	C	V	594
GAT	GTG	AAC	GAG	TGC	GAG	GCA	GAG	CCC	TGC	GGC	CCC	GGG	AAA	GGC	ATC	TGT	ATG	1836
D	V	N	E	C	E	A	E	P	C	G	P	G	K	G	I	C	M	612
AAC	ACT	GGT	GGC	TCC	TAC	AAT	TGT	CAC	TGC	AAC	CGA	GGC	TAC	CGC	CTC	CAC	GTG	1890
N	T	G	G	S	Y	N	C	H	C	N	R	G	Y	R	L	H	V	630
GGT	GCA	GGG	GGC	CGC	TCG	TGC	GTG	GAC	CTG	AAC	GAG	TGC	GCC	AAG	CCT	CAC	CTG	1944
G	A	G	G	R	S	C	V	D	L	N	E	C	A	K	P	H	L	648
TGT	GGG	GAC	GGT	GGC	TTC	TGC	ATC	AAC	TTC	CCT	GGT	CAC	TAC	AAA	TGC	AAC	TGC	1998
C	G	D	G	G	F	C	I	N	F	P	G	H	Y	K	C	N	C	666
TAT	CCT	GGC	TAC	CGG	CTC	AAG	GCC	TCC	CGA	CCG	CCC	ATT	TGC	GAA	GAC	ATC	GAC	2052
Y	P	G	Y	R	L	K	A	S	R	P	P	I	C	E	D	I	D	684
GAG	TGT	CGC	GAC	CCT	AGC	ACC	TGC	CCT	GAT	GGC	AAA	TGT	GAA	AAC	AAA	CCT	GGC	2106
E	C	R	D	P	S	T	C	P	D	G	K	C	E	N	K	P	G	702

FIG. 25-2

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AGC TTC AAG TGC ATC GCC TGC CAG CCT GGC TAC CGT AGC CAG GGG GGC GGG GCC 2160
 S F K C I A C Q P G Y R S Q G G G A 720
 TGT CGT GAT GTC AAC GAA TGC TCC GAA GGT ACC CCC TGC TCT CCT GGA TGG TGT 2214
 C R D V N E C S E G T P C S P G W C 738
 GAG AAA CTT CCG GGT TCT TAC CGT TGC ACG TGT GCC CAG GGG ATA CGA ACC CGC 2268
 E K L P G S Y R C T C A Q G I R T R 756
 ACA GGA CGC CTC AGT TGC ATA GAC GTG GAT GAC TGT GAG GCT GGG AAA GTG TGC 2322
 T G R L S C I D V D D C E A G K V C 774
 CAA GAT GGC ATC TGC ACG AAC ACA CCA GGC TCT TTC CAG TGT CAG TGC CTC TCC 2376
 Q D G I C T N T P G S F Q C Q C L S 792
 GGC TAT CAT CTG TCA AGG GAT CGG AGC CGC TGT GAG GAC ATT GAT GAA TGT GAC 2430
 G Y H L S R D R S R C E D I D E C D 810
 TTC CCT GCG GCC TGC ATC GGG GGT GAC TGC ATC AAT ACC AAT GGT TCC TAC AGA 2484
 F P A A C I G G D C I N T N G S Y R 828
 TGT CTC TGT CCC CTG GGT CAT CGG TTG GTG GGC GGC AGG AAG TGC AAG AAA GAT 2538
 C L C P L G H R L V G G R K C K K D 846
 ATA GAT GAG TGC AGC CAG GAC CCA GGC CTG TGC CTG CCC CAT GCC TGC GAG AAC 2592
 I D E C S Q D P G L C L P H A C E N 864
 CTC CAG GGC TCC TAT GTC TGT GTC TGT GAT GAG GGT TTC ACA CTC ACC CAG GAC 2646
 L Q G S Y V C V C D E G F T L T Q D 882
 CAG CAT GGG TGT GAG GAG GTG GAG CAG CCC CAC CAC AAG AAG GAG TGC TAC CTT 2700
 Q H G C E E V E Q P H H K K E C Y L 900
 AAC TTC GAT GAC ACA GTG TTC TGT GAC AGC GTA TTG GCT ACC AAT GTC ACT CAG 2754
 N F D D T V F C D S V L A T N V T Q 918
 CAG GAA TGC TGT TGC TCT CTG GGA GCT GGC TGG GGA GAC CAC TGC GAA ATC TAT 2808
 Q E C C C S L G A G W G D H C E I Y 936
 CCC TGT CCA GTC TAC AGC TCA GCC GAA TTT CAC AGC CTG GTG CCT GAT GGG AAA 2862
 P C P V Y S S A E F H S L V P D G K 954
 AGG CTA CAC TCA GGA CAA CAA CAT TGT GAA CTA TGC ATT CCT GCC CAC CGT GAC 2916
 R L H S G Q Q H C E L C I P A H R D 972
 ATC GAC GAA TGC ATA TTG TTT GGG GCA GAG ATC TGC AAG GAG GGC AAG TGT GTG 2970
 I D E C I L F G A E I C K E G K C V 990
 AAC TCG CAG CCC GGC TAC GAG TGC TAC TGC AAG CAG GGC TTC TAC TAC GAT GGC 3024
 N S Q P G Y E C Y C K Q G F Y Y D G 1008
 AAC CTG CTG GAG TGC GTG GAC GTG GAC GAG TGC TTG GAT GAG TCT AAC TGC AGG 3078
 N L L E C V D V D E C L D E S N C R 1026
 AAC GGA GTG TGT GAG AAC ACG TGG CGG CTA CCG TGT GCC TGC ACT CCG CCG GCA 3132
 N G V C E N T W R L P C A C T P P A 1044
 GAG TAC AGT CCC GCA CAG GCC CAG TGT CTG AGC CCG GAG GAG ATG GAG CAC GCC 3186
 E Y S P A Q A Q C L S P E E M E H A 1062

FIG. 25-3

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CCA GAG AGA CGT GAA GTG TGC TGG GGC CAG CGA GGA GAG GAC GGC ATG TGT ATG 3240
 P E R R E V C W G Q R G E D G M C M 1080
 GGG CCC CTG GCG GGA CCT GCC CTC ACT TTT GAT GAC TGC TGC TGC CGC CAG CCG 3294
 G P L A G P A L T F D D C C C R Q P 1098
 CGG CTG GGG TAC CAG TGC AGA CCG TGC CCG CCA CGT GGC ACC GGG TCC CAG TGC 3348
 R L G Y Q C R P C P P R G T G S Q C 1116
 CCG ACT TCA CAG AGT GAG AGC AAT TCT TTC TGG GAC ACA AGC CCC CTG CTA CTG 3402
 P T S Q S E S N S F W D T S P L L L 1134
 GGG AAG TCT CCG CGA GAC GAA GAC AGC TCA GAG GAG GAT TCA GAT GAG TGC CGT 3456
 G K S P R D E D S S E E D S D E C R 1152
 TGT GTG AGC GGA CCG TGT GTG CCA CGG CCA GGC GGG GCG GTA TGC GAG TGT CCT 3510
 C V S G P C V P R P G G A V C E C P 1170
 GGA GGC TTT CAG CTG GAC GCC TCC CGT GCC CGC TGC GTG GAC ATT GAT GAG TGC 3564
 G G F Q L D A S R A R C V D I D E C 1188
 CGA GAA CTG AAC CAG CGG GGA CTG CTG TGT AAG AGC GAG CGG TGC GTG AAC ACC 3618
 R E L N Q R G L L C K S E R C V N T 1206
 AGT GGA TCC TTC CGC TGT GTC TGC AAA GCT GGC TTC ACG CGC AGC CGC CCT CAC 3672
S G S F R C V C K A G F T R S R P H 1224
 GGG CCT GCG TGC CTC AGC GCC GCC GCT GAT GAT GCA GCC ATA GCC CAC ACC TCA 3726
 G P A C L S A A A D D A A I A H T S 1242
 GTG ATC GAT CAT CGA GGG TAT TTT CAC TGA
 V I D H R G Y F H *

FIG. 25-4

SUBSTITUTE SHEET (RULE 26)

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Met Arg Gln Ala Ala Leu Gly Leu Leu Ala Leu Leu Leu Ala Leu Leu Gly Pro Gly Gly Arg	22
Gly Val Gly Arg Pro Gly Ser Gly Ala Gln Ala Gly Ala Gly Arg Trp Ala Gln Arg Phe Lys Val	44
Val Phe Ala Pro Val Ile Cys Lys Arg Thr Cys Leu Lys Gly Gln Cys Arg Asp Ser Cys Gln Gln	66
Gly Ser Asn Met Thr Leu Ile Gly Leu Pro Cys Met Asn Gly His Ser Thr Asp Thr Leu Thr Gly Ser Ala Phe	88
Arg Val Val Val Cys Pro Leu Cys Phe Thr Gly Arg Phe Cys Met Asn Gly Gln Cys Ser Ser Arg Asn Gln Cys Leu	110
Cys Pro Pro Asp Phe Thr Gly Arg Phe Cys Gln Val Pro Ala Ala Gly Thr Gly Ala Gly Thr Gly	132
Ser Ser Gly Pro Gly Trp Pro Asp Arg Ala Met Ser Thr Gly Pro Leu Pro Pro Leu Ala Pro Glu	154
Gly Glu Ser Val Ala Ser Lys His Ala Ile Tyr Ala Val Gln Val Ile Ala Asp Pro Pro Gly Pro	176
Gly Glu Gly Pro Pro Ala Gln His Ala Phe Leu Val Pro Leu Gly Pro Gly Gln Ile Ser Ala	198
Glu Val Gln Ala Pro Pro Val Val Asn Val Arg Val His His Pro Pro Glu Ala Ser Val Gln	220
Val His Arg Ile Glu Gly Pro Asn Ala Glu Gly Pro Ala Ser Ser Gln His Leu Leu Pro His Pro	242
Lys Pro Pro His Pro Arg Pro Pro Thr Gln Lys Pro Leu Gly Arg Cys Phe Gln Asp Thr Leu Pro	264
Lys Gln Pro Cys Gly Ser Asn Pro Leu Pro Gly Leu Thr Lys Gln Glu Asp Cys Cys Gly Ser Ile	286
Gly Thr Ala Trp Gly Gln Ser Lys Cys His Lys Cys Pro Gln Leu Gln Tyr Thr Gly Val Gln Lys	308
Pro Val Pro Val Arg Gly Glu Val Gly Ala Asp Cys Pro Gln Gly Tyr Lys Arg Leu Asn Ser Thr	330
His Cys Gln Asp Ile Asn Glu Cys Ala Met Pro Gly Asn Val Cys His Gly Asp Cys Leu Asn Asn	352
Pro Gly Ser Tyr Arg Cys Val Cys Pro Pro Gly His Ser Leu Gly Pro Leu Ala Ala Gln Cys Ile	374
Ala Asp Lys Pro Glu Glu Lys Ser Leu Cys Phe Arg Leu Val Ser Thr Glu His Gln Cys Gln His	396
Pro Leu Thr Thr Arg Leu Thr Arg Gln Leu Cys Cys Ser Val Gly Lys Ala Trp Gly Ala Arg	418
Cys Gln Arg Cys Pro Ala Asp Gly Thr Ala Ala Phe Lys Glu Ile Cys Pro Gly Trp Glu Arg Val	440
Pro Tyr Pro His Leu Pro Pro Asp Ala His His Pro Gly Gly Lys Arg Leu Leu Pro Leu Pro Ala	462
Pro Asp Gly Pro Pro Lys Pro Gln Gln Leu Pro Glu Ser Pro Ser Arg Ala Pro Pro Leu Glu Asp	484

FIG. 26-1

SUBSTITUTE SHEET (RULE 26)

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Thr Glu Glu Thr Arg Gly Val Thr Met Asp Pro Pro Val Ser Glu Glu Arg Ser Val Gln Gln Ser 506
 His Pro Thr Thr Thr Ser Pro Pro Arg Pro Tyr Pro Glu Leu Ile Ser Arg Pro Ser Pro Pro 528
 Thr Phe His Arg Phe Leu Pro Asp Leu Pro Pro Ser Arg Ser Ala Val Glu Ile Ala Pro Thr Gln 550
 Val Thr Glu Thr Asp Glu Cys Arg Leu Asn Gln Asn Ile Cys Gly His Gly Gln Cys Val Pro Gly 572
 Pro Ser Asp Tyr Ser Cys His Cys Asn Ala Gly Tyr Arg Ser His Pro Gln His Arg Tyr Cys Val 594
 Asp Val Asn Glu Cys Glu Ala Glu Pro Cys Gly Pro Gly Lys Gly Ile Cys Met Asn Thr Gly Gly 616
 Ser Tyr Asn Cys His Cys Asn Arg Gly Tyr Arg Leu His Val Gly Ala Gly Arg Ser Cys Val 638
 Asp Leu Asn Glu Cys Ala Lys Pro His Leu Cys Gly Asp Gly Gly Phe Cys Ile Asn Phe Pro Gly 660
 His Tyr Lys Cys Asn Cys Tyr Pro Gly Tyr Arg Leu Lys Ala Ser Arg Pro Pro Ile Cys Glu Asp 682
 Ile Asp Glu Cys Arg Asp Pro Ser Thr Cys Pro Asp Gly Lys Cys Glu Asn Lys Pro Gly Ser Phe 704
 Lys Cys Ile Ala Cys Gln Pro Gly Tyr Arg Ser Gln Gly Gly Ala Cys Arg Asp Val Asn Glu 726
 Cys Ser Glu Gly Thr Pro Cys Ser Pro Gly Trp Cys Glu Lys Leu Pro Gly Ser Tyr Arg Cys Thr 748
 Cys Ala Gln Gly Ile Arg Thr Arg Thr Gly Arg Leu Ser Cys Ile Asp Val Asp Cys Glu Ala 770
 Gly Lys Val Cys Gln Asp Gly Ile Cys Thr Asn Thr Pro Gly Ser Phe Gln Cys Gln Cys Leu Ser 792
 Gly Tyr His Leu Ser Arg Asp Arg Ser Arg Cys Glu Asp Ile Asp Glu Cys Asp Phe Pro Ala Ala 814
 Cys Ile Gly Gly Asp Cys Ile Asn Thr Asn Gly Ser Tyr Arg Cys Leu Cys Pro Leu Gly His Arg 836
 Leu Val Gly Gly Arg Lys Cys Lys Asp Ile Asp Glu Cys Ser Gln Asp Pro Gly Leu Cys Leu 858
 Pro His Ala Cys Glu Asn Leu Gln Gly Ser Tyr Val Cys Val Cys Asp Glu Gly Phe Thr Leu Thr 880
 Gln Asp Gln His Gly Cys Glu Glu Val Glu Gln Pro His His Lys Lys Glu Cys Tyr Leu Asn Phe 902
 Asp Asp Thr Val Phe Cys Asp Ser Val Leu Ala Thr Asn Val Thr Gln Gln Glu Cys Cys Ser 924
 Leu Gly Ala Gly Trp Gly Asp His Cys Glu Ile Tyr Pro Cys Pro Val Tyr Ser Ser Ala Glu Phe 946
 His Ser Leu Val Pro Asp Gly Lys Arg Leu His Ser Gly Gln Gln His Cys Glu Leu Cys Ile Pro 968

FIG. 26-2

SUBSTITUTE SHEET (RULE 26)

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Ala His Arg Asp Ile Asp Glu Cys Ile Leu Phe Gly Ala Glu Ile Cys Lys Glu Gly Lys Cys Val	990
Asn Ser Gln Pro Gly Tyr Glu Cys Tyr Cys Lys Gln Gly Phe Tyr Tyr Asp Gly Asn Leu Leu Glu	1012
Cys Val Asp Val Asp Glu Cys Thr Pro Pro Arg Asp Glu Ser Asn Cys Arg Asn Gly Val Cys Glu Asn Thr Trp	1034
Arg Leu Pro Cys Ala Cys Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser Glu Glu Arg Thr Ala Cys	1056
Glu Arg Trp Ser Thr Pro Gln Arg Asp Val Lys Cys Ala Gly Ala Ser Glu Glu Arg Thr Ala Cys	1078
Val Trp Gly Pro Trp Ala Gly Pro Ala Leu Thr Phe Asp Asp Cys Cys Arg Gln Pro Arg Leu	1100
Gly Thr Gln Cys Arg Pro Cys Pro Pro Arg Gly Thr Gly Ser Gln Cys Pro Thr Ser Gln Ser Glu	1122
Ser Asn Ser Phe Trp Asp Thr Ser Pro Leu Leu Leu Leu Gly Lys Ser Pro Arg Asp Glu Asp Ser Ser	1144
Glu Glu Asp Ser Asp Glu Cys Arg Cys Val Ser Gly Pro Cys Val Pro Arg Pro Gly Ala Val	1166
Cys Glu Cys Pro Gly Gly Phe Gln Leu Asp Ala Ser Arg Ala Arg Cys Val Asp Ile Asp Glu Cys	1188
Arg Glu Leu Asn Gln Arg Gly Leu Leu Cys Lys Ser Glu Arg Cys Val Asn Thr Ser Gly Ser Phe	1210
Arg Cys Val Cys Lys Ala Gly Phe Thr Arg Ser Arg Pro His Gly Pro Ala Cys Leu Ser Ala Ala	1232
Ala Asp Asp Ala Ala Ile Ala His Thr Ser Val Ile Asp His Arg Gly Tyr Phe His	1251

FIG. 26-3

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ATGAGAGCA CCTCCCGCG AGGTCTCCG TGCCACAGC TCTGCAGCA CTCTGGCGC ATGAGAGCG CGACCACCG 80
 TCGTGTCTCC GGATGCATCC AACGGGTGCG TTGGAGGGG TTCTGTCCAC TTGTCTTGATG GGGACAAGTC 160
 ATGCCCAACG GGATTCCATA GGGAGATACG AACCACTAG AACCGATCG AATCGTTGT GGCACCCCGT GGGCAGCCAC 240
 CCGCAGCGG CTGCAGCCAA GGTGTACAGT CTGTTCCGAG AGCCTGACGC GCCGTGCCC GGCTTGTGCG CCTCTGAGTG 320
 GAACCAAGCG GCCCAGGGGA ACCCGGGATG GCTCGCAGAG GCCGAGGCC GAGGCCACC TCGAACCCAG CAGCTGCGTC 400
 GAGTCCAGCC ACCTGTCCAG ACTCGGAGAA GCCATCCCG GGGCCAGCAG CAGATAGCAG CCGGGCTGC ACCTTCTGTC 480
 GCGCGCTGG AACCCCTCA GCGACCCGCG GCTGCACGGC GAGGGCGGT CACTGGGAGA AATGTCTGCG GGGACAGTG 560
 CTGCCCAGGA TGGACAACAT CAACACGAC CAACCACTGT ATCAACCTG TGTGTCAGC TCCCTGTCAG AACCGAGGT 640
 CCTGCAGCAG GCCCAGGTC TGCATCTGCC GTTCTGGCTT CCGTGGGCG CGCTGTGAGG AGGTCTATCC TGAGGAGAA 720
 TTTGACCTC AGAATGCCAG GCCTGTGCCC AGACGCTCAG TGGAGAGAG ACCGGTCTT CACAGAAGCA GTGAGGCCAG 800
 AGGAAGTCTA GTGACCAGAA TACAGCCGCT GGTACCACCA CCATCACCA CTCCATCTCG GCGCCTCAGC CAGCCCTGGC 880
 CCTGTCAGCA GCACTCAGG CCGTCCAGGA CAGTTGCTCG GTATCCGGC ACTGGTGCCA ATGGCCAGT GATGTCCAAC 960
 GCTTTGCCCT CAGGACTCGA GCTGAGAGAC AGCAGCCAC AGCAGACCA TGTGAACCAT CTCTCACCCC CCTGGGGCT 1040
 GAACCTCACC GAGAAATCA AGAAATCAA AGTGTCTTC ACCCCACCA TCTGCAAGCA GACCTGTGCG CCGGGACGCT 1120
 GTGCCAACAG CTGTGAGAG GGTGACACCA CCACCTTGA CAGTCAGGT GGCCATGGG ATGACCCCAA GTCTGGCTTC 1200
 CGTATCTATT TCTGCCAAT CCCCTGCCCTG AATGGTGCC GCTGCATCG CCGGGACGAG TGTGTGTGTC CAGCCAATC 1280
 CACAGGAAAG TTCTGCCATC TGCCCTGTCG GCAGCCAGAC AGGAACTG CAGGGCGAGG TTCCCGGCAC AGAACCTGC 1360
 TGGAGGTCC CTTGAAGCAA TCCACCTTCA CGCTGCCTCT CTCTAACCA CTGCGCTCTG TGAACCCCTC GCTGGTGAAG 1440
 GTGCAATTC ATACCCGCC TGAGGCCTCT GTGCAGATT ACCAGGTGG CCGGTCCCG GGTGAGCTGG ACCCGTGTCT 1520
 GGAGGACAC AGTGTGGAGA CCAGAGCCTC TCATCGCCCC CACGGCAACC TAGGCCACAG CCCCTGGGCC AGCAACAGCA 1600
 TACCCGCTCG GCGCGGAGAG GCGCCTCGG CACCACAGT GCTGTCTAG CATTATGGAC TTCTGGGCCA GTGTTACCTG 1680
 AGCACGGTGA ATGACAGTG TGCTAACCC CTAGGTAGTC TGACTTCTCA GGAGGACTGC TGTGGCAGTG TGGGACCTT 1760
 CTGGGGGTG ACCTCCTGTG CTCCTGTGCC ACCCAGACAA GAGGGTCCAG CCTTCCAGT GATTGAAAT GGCCAGCTGG 1840
 AGTGTCCCA AGGATACAAG AGACTGAAC TCAGCCACTG CCAAGATATC AATGAGTGC TGAACCTGGG CCTCTGCAAG 1920

SUBSTITUTE SHEET (RULE 26)

FIG. 27-1

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GACTCGGAGT GCGTGAACAC CAGGGGCGAG TACTGTGTGA CCTGCAGGCC TGGCCTCATG CTGGATCCGT CAAGGAGCGG 2000
 CTGCGTATCG GACAAGGCTG TCTCCATGCA GCAGGGAATA TGCTACCGGT CACTGGGGTC TGGTACCTGC ACCCTGCCCTT 2080
 TGGTTTCATCG GATCACCAG CAGATATGCT GCTGCAGCG TGTGGGCAAA GCCTGGGGTA GCACATGTGA ACAGTGTCCC 2160
 CTGCTGGCA CAGAAGCCTT CAGGGAGATC TGCCCTGTCTG GCCATGGCTA CACCTACTCG AGCTCAGACA TCCGCCCTGTC 2240
 TATGAGGAAA GCCGAAGAAG AGGAACCTGG TAGCCCTTTA AGGGAGCAGA CAGAGCAGAG CACTGCACCC CCACCTGGGC 2320
 AAGCAGAGAG GCAACCACTC CCGGCAAGCA CCGCCACCTG GATTGAGGCT GAGACCTCC CTGACAAAAGG TGACTCTCGG 2400
 GCTGTTTACA TCACAACCAG TGCTCCCCAC CTACCTGCCC GGGTACCAGG GGATGCCACT GGAAGACCAG CACCATCCTT 2480
 GCCTGGACAG GGCATTCCAG AGAGTCCAGC AGAAGAGCAA GTGATTCCCT CCAGTGATGT CTTGGTGACA CACAGCCCCC 2560
 CAGACTTTGA TCCATGTTTT GCTGGAGCCT CCAACATCTG TGGCCCTGGG ACCTGTGTGA GCCTCCCAA TGGATACAGA 2640
 TGTGCTGCA GCCCTGGCTA CCAGCTACAC CCAGCCAAG ACTACTGTAC TGATGACAAC GAGTGTATGA GGAACCCCTG 2720
 TGAAGGAAGA GGGCGCTGTG TCAACAGTGT GGGCTCCTAC TCCTGCCCTCT GCTATCCTGG CTACACACTA GTCACCTCG 2800
 GAGACACACA GGAGTGCCAA GATATCGATG AGTGTGAGCA GCCCGGGTG TGCAGTGGTG GCGATGCAG CAACACGGAG 2880
 GGCTCGTACC ACTGCGAGTG TGATCGGGC TACATCATGG TCAGGAAAGG ACACGTGCAA GATATCAACG AATGCCGTCA 2960
 CCCTGGTACC TGCCCTGATG GGAGATGCCGT CAACTCCCCT GGCTCCTACA CTTGTCTGGC CTGTGAGGAG GGCTATGTAG 3040
 GCCAGAGTGG GAGCTGTGTA GATGTCAATG AGTGTCTGAC CCCTGGGATA TGTACCCATG GAAGGTGCAT CAACATGGAA 3120
 GGCTCCTTTA GATGCTCCTG TGAGCCGGG CATTCAACAG CCGGCTCCT TCACCTGCTC AGCCTGTCTG ACAGTGTGC 3200
 CAGCCGAGCC TCGTGCCCCA CCGGCTCTG CCTCAACAG GAGGGCTCCT TCACCTGCTC AGCCTGTCTG AGCGGTACT 3280
 GGGTGAACGA AGATGGCACT GCCTGTGAAG ACTTGGATGA ATGTGCCTTC CCTGGAGTCT GCCCCACAGG CGTCTGCACC 3360
 AATACTGTAG GCTCCTTCTC CTGCAAGGAC TGTGACCAGG GCTACCGGCC CAACCCCTG GGCAACAGAT GCCTCTGTC 3440
 GGATGAGTGT GAAAGTCCCC AAAGCAGCTG CCGGGGAGGC GAATGCAAGA ACACAGAAGG TTCTTACCAA TGCCTCTGTC 3520
 ACCAGGGCTT CCAGTGGTC AATGGCACCA TGTGTGAGGA CGTGAATGAG TGTGTTGGG AAGAGCATTTG TGCTCCTCAC 3600
 GCGAGTGC TCAACAGCCT GGGCTCCTTC TTCTGCCCTCT GTGCACCCGG CTTTGTCTAGT GCTGAGGGGG GCACCAGATG 3680
 CCAGGATGTT GATGAATGTG CAGCCACAGA CCCGTGTCCG GGAGGACACT GTGTCAACAC AGAGGGCTCC TTCAGCTGTC 3760
 TGTGTGAGAC TGCTTCTCTC CAGCCCTCCC CAGACAGCGG AGAATGTTTG GATATTGATG AGTGTGAGGA CCGTGAAGAC 3840

SUBSTITUTE SHEET (PRIORITY)

FIG. 27-2

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CCGGTGTGCG GAGCCTGGAG GTGTGAGAAC AGTCTTGTTT CCTACCGCTG CATCTGGAC TGCCAGCCTG GATTCTATGT 3920
 GCGGCCAAAT GGAGACTGCA TTGACATAGA TGAATGTGCC AATGACACTG TGTGTGGAA CCATGGCTTC TGTGACAAACA 4000
 CGGACGGCTC CTTCCGGCTGC CTGTGTGACC AGGGCTTCGA GACCTCACCA TCAGGCTGGG AGTGTGTTGA TGTGAACGAG 4080
 TGTGAGCTCA TGATGGCAGT GTGTGGGGAT GCGCTCTGTG AGAACGTGGA AGGCTCCTTC CTGTGCTTTT GCGCCAGTGA 4160
 CCTTGAGGAG TACGACGCAG AAGAAGGACA CTGCCGTCCT CGGCTGGCTG GAGCTCAGAG AATCCCAGAG GTCCGGACAG 4240
 AGGACCAGGC TCCAAGCCTT ATCCGCATGG AATGCTACTC TGAACACAAAT GGTGTCTCTC CCTGCTCTCA AATCCTGGGC 4320
 CAGAACTCCA CACAGGCCGA GTGCTGTGTC ACTCAGGCTG CCAGATGGGG AAAGCCTGT GCGCCCTGCC CATCTGAGGA 4400
 CTCAGTTGAA TTCAGTCAGC TCTGCCCCAG TGGTCAAGGT TACATCCAG TGAAGGAGC CTGGACATTT GGACAAACCA 4480
 TGTATACAGA TGCCGATGAA TGTGTACTGT TTGGGCTGC TCTGTGCCAG AATGGCCGAT GCTCAAAACAT AGTGCCTGGC 4560
 TACATTTGCC TGTGCAACCC TGGCTACCC TATGATGCCT CCAGCAGGAA GTGCCAGGAT CACAACGAAT GCCAGGACTT 4640
 GGCCTGTGAG AACGGTGAGT GTGTGAACCA AGAAGGCTCC TTCCATTGCC TCTGCAATCC CCCCCTCACC CTAGACCTCA 4720
 GTGGGCAGCG CTGTGTGAAC ACGACCAGCA GCACGGAGGA CTTCCCTGAC CATGACATCC ACATGGACAT CTGCTGGAAA 4800
 AAAGTCACCA ATGATGTGTG CAGCCAGCCC TTGCCGTGGC ACCATACCAC CTATACAGAA TGCTGCTGCC AAGATGGGA 4880
 GGCCTGGAGC CAGCAATGCG CTCTGTGCCC GCCCAGGAGC TCTGAGGTCT ACGCTCAGCT GTGCAACGTG GCTCGGATTG 4960
 AGGCAGAGCG CGGAGCAGGG ATCCACTTCC GGCCAGGCTA TGAGTATGGC CCTGGCCTGG ACGATCTGCC TGAACACCTC 5040
 TACGGCCCCAG ATGGGGCTCC CTTCTATAAC TACCTAGGCC CCGAGGACAC TGCCCCCTGAG CCTCCCTTCT CCAACCCAGC 5120
 CAGCCAGCCG GGAGACAACA CACCTGTCTT TGAGCCTCCT CTGACGCCCT CTGAACCTCA GCCTCACTAT CTAGCCAGCC 5200
 ACTCAGAAC CCCTGCCTCC TTCGAAGGCC TTCAGGCTGA GGAATGTGGC ATCCTGAATG GCTGTGAGAA TGGCCGCTGC 5280
 GTGCGTGTGC GGGAGGGCTA CACTTGGGAC TGCTTTGAGG GCTTCCAGCT GGATGGCCCC ACATTGGCCT GTGTGGATGT 5360
 GAACGAGTGT GAAGACTTGA ACGGGCCTGC ACGACTCTGT GCACACGGTC ACTGTGAGAA CACAGAGGGT TCCTATCGCT 5440
 GCCACTGTTT GCCAGGTTAC GTGGCAGAGC CAGGCCCCCC AACTGTGCG GCCAAGGAGT AG 5502

SUBSTITUTE SHEET (RULE 26)

FIG. 27-3

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MESTSPRGLRCPQLCSHSGAMRAPTTARCSGCIQVRWRGFLPLVLAVLMTSHAQDSIGRYEPASRDANRLWHPVSHPA AAAKVYS 90
 LFREPDAPVPGLSPEWNQPAQGNPGWLAEAEARRPPRTQQLRRVQPPVQTRRSHPRGQQIAARAAPSVARLETQRPAAARRGLTGR 180
 NVCGGQCCPGWTTSTNSNTHCIKPVCCQPCQNRGSCSRPQVICRSGRGARCEEVPEEEFDPQONARPVPRRSVERAPGPHRSSEARGSL 270
 VTRIQLPLVPPPPPPRRRLSQPWLQOHSGSPSRTVRRYPATGANGQLMSNALPSGLELRDSSPQAAHVNLSPPWGLNLTEKIKKVVVF 360
 TPTICKQTCARGRCANSCCKGDTTTLYSQGGHGHDPKSGFRIYFCQIPCLNGGRCIGRDECWCPANSTGKFCHLPVPQPDREPAGRSRH 450
 RTLLEGPLKQSTFTPLPLSNQLASVNP SLVKVQIHHPPPEASVQIHQVARVRGELDPVLEDNSVETRASHRPHGNLGHSPWASNSIPARAGE 540
 APRPPVLSRHYGLLGQCYLSTVNGQCANPLGSLTSEDCCSGSVGTFWGVTSCAPCPPRQEGPAPFVIENGQLECPQGYKRLNLSHCQDI 630
 NECLTLGLCKDSECVNTRGSYLCTCRPGLMLDPSRSRCVSDKAVSMQQLCYRSLSGTCTTLPLVHRITKQICCCSRVKGAWGSTCEQCP 720
 LPGTEAFREICPAGHGTYSSDIRLSMRKAEELASPLREQTEQSTAPPQQAERQPLRAATATWIEAETLPDKGDSRAVQITTSAPH 810
 LPARVPGDATGRPAPSLPGQIPESPAEEQVIPSSDVLVTHSPDPDFPCFAGASNICGPGTCVSLPNGYRCVCSPGYQLHPSQDYCTDDN 900
 BCMRNPCEGRGRCVNSVGSYCLCYPGYTLVLTGDTQECQIDICEQPGVCSGGRCNTEGSHCECDRGYIMVRKGHCQDINECRHPGT 990
 CPDGRCVNSPGSYTCLACEGYVQSGSCVDVNECLTPGICTHGRGINMEGSRFCSEPEGYEVTDDKGRDVEDCASRASCP TGLCLNT 1080
 EGSFTCSACQSGYWNEDGTACEDLDECAFPQVCTGVTNTVGSFCKDCDQGYRPNPLGNRCEDVDECEGPQSSCRGGECKNTEGSYQ 1170
 CLCHQGFQLVNGTMCEDVNECVGEEHCAPHGECINSLGSFFCLCAPGFASAEAGGTRCQDVDECAATDPCPGGHCVNTEGFSCLCETASF 1260
 QPSDPSGECLDIDECEDREDPVCGAWRCENSPGSRRCILDCQPGFYVAPNGDCIDIDECANDTVCGNHGFCNDTSGFRCLCDQGFETSP 1350
 SGWECVDVNECELMMAVCGDALCENVEGSSFLCLCASDLEEDAEEGHCRPRVAGAQRIPEVRTEDQAPSLIRMECYSEHNGGPPCSQILG 1440
 QNSTQAECCCTQGARWKGACAPCPSEDSVEFSQLCPSGQGYIPVEGAWTFGTMYTDAECVLFGPALCQNGRCNSIVPGYICLCNPGYH 1530
 YDASSRKCCQDHNEDQDLACENGECVNGEGSFHCLCNPPITLTLDSGQRCVNTTSTEDFPDHDIHMDICWKKVTNDVCSQPLRGHHTTYTE 1620
 CCCQDGEAWSQOCCALCPPRSSEVYAQLCNVARIEAERGAGIHFRPGYEGPGLDDLLENLYGPDGAPFYNYLGPEDTAPPEPFSNPASQP 1710
 GDNTPVLEPPLQPSSELQPHYLASHSEPPASFEGLOAECCGILNGCENGRCVRREGYTCDCFEGFQLDAPTLACVDVNECEDLNGPARLC 1800
 AHGHCENTEGSYRCHCSPGYVAEPGPPHCAAKE 1833

FIG. 28

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